ANTI-PROLIFERATIVE WITHANOLIDES FROM *VASSOBIA BREVIFLORA* AND *WITHANIA SOMNIFERA*

By

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ABSTRACT

In order to identify anti-proliferative compounds from selected Latin American plants, approximately 200 plant extracts were screened by the MTS assay (3-[4,5-dimethylthiazol-2yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2*H* tetrazolium). *Vassobia breviflora* (Solanaceae), was identified as the most active of the species tested in this study. Following a bioassay-guided approach, withaferin A was isolated and characterized. This withanolide-type steroidal lactone showed anti-proliferative activities with IC50 values from 0.5 to 2.2 μM against head and neck squamous cell carcinoma (HNSCC) MDA1986, JMAR, UM-SCC-2 and JHU011. A mechanistic study showed that withaferin A induced apoptosis and cell death in HNSCC cells as well as a shift from G0/G1 to G2/M arrest in cell cycle studies. Western data demonstrated that the anti-proliferative action of withaferin A could be in part explained through degradation of total Akt levels as well as decrease in activation of Akt levels. In order to establish anticancer structure activity relationships, three analogues were semi-synthesized from withaferin A, and ten related withanolides, including a new chlorinated withanolide 6*α*-chloro-5*β*,17*α*-dihydroxywithaferin, were isolated from *Withania somnifera* (Solanaceae). All structures were elucidated on the basis of spectroscopic methods (IR, MS, and 1D/2D NMR). X-ray crystallography confirmed the absolute configuration of the new withanolide. A method for large-scale isolation of withaferin A from *W. somnifera* was also developed and presented.

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I. INTRODUCTION

The use of medicines from nature has a long history of development and application by humans. Plant medicines have been well documented in ancient Egypt, India, China, Greece and Rome. One of the earliest Egyptian medicine records, the "Edwin Smith Papyrus", is dated circa 3000 BCE.[1] In India, traditional medicine composed of different system such as Ayurveda, Siddha, Unani, homeopathy and naturopathy, has been thriving for many centuries.[2] China also has a rich heritage of traditional plant medicine, which dates back more than 4,000 years. Due to their long historical use and reliable therapeutic efficacy, traditional plant medicines are still widely used in these countries.

Medicinal plants are also gaining more attention from contemporary pharmaceutical academic institutes, as they are an important source for the discovery and development of new natural bioactive compounds, and provide a diversity of lead structures for the development of synthetic and/or semi-synthetic analogs. In Newman and Cragg's review, 63% of the 974 new chemical entities introduced from January 1981 to December 2007 were natural products, modified natural products or synthetic compounds with a natural product pharmacophore. When considering cancer chemotherapies, among 196 anticancer drugs introduced, 79.8% were naturally derived or inspired.[3] For example, Paclitaxel, which was first isolated and characterized from the Pacific Yew tree *Taxus brevifolia* in 1971 by Wani and colleagues in North Carolina,[4] is clinically used against breast, lung and ovarian cancer. It is the best-selling cancer drug ever manufactured.

Camptothecin is another widely used plant derived anticancer agent, which is a cytotoxic quinoline alkaloid first isolated and identified from *Camptotheca acuminata* in 1966 by Wall and colleages.[5]

Over 1.7 million of the world's species of animals, plants and algae have been described in the IUCN red list of threatened species, 2010.[6] According to the estimates made by scientists, 5 million to over 50 million species live on the planet earth.[7] However, among *ca.* 320,000 plant species recorded, only a small portion has been explored. Marine organisms and microorganisms are nearly undiscovered. Therefore, finding new drug leads from natural origins is still one of the methods followed in drug lead discovery, especially for cancer research.

Medicinal plants usually contain complex constituents, such as fatty acids, polyketides, terpenoids, steroids, and alkaloids, so the evaluation, standardization and quantification of the active ingredients are usually difficult to determine in whole plant samples. Efficient methods are therefore required for the separation and identification of these active ingredients from other components in plants. The field of science to study physical, chemical, biochemical and biological properties of drugs, drug substances, or potential drugs or drug substances of natural origin as well as the search for new drugs from natural sources, is defined as pharmacognosy by the American Society of Pharmacognosy. The purpose of this thesis work was to search for anti-proliferative compounds from selected Latin American plants that have not been previously investigated in detail.

The classical phytochemical methods for finding bioactive natural products are to isolate the compound first, then to screen its particular activities *in vitro* or *in vivo* through different types of bioassays. Chemical and chromatographic techniques used in isolation and purification processes include liquid-liquid phase extraction, thin-layer chromatography (TLC), preparative TLC (PTLC), column chromatography, medium pressure liquid chromatography (MPLC), high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectroscopy (LC-MS). Spectroscopic methods such as IR, 1D and 2D NMR, and UV are used for identifying the isolated compounds.

Isolation and identification of compounds from plants is a time consuming activity while many pure compounds isolated may show no activity whatsoever after the biological assays are performed. In this research project, a bioassay guided fractionation was applied for searching anticancer bioactive compounds. Instead of screening the pure compounds from two genera in the family Solanaceae, fractions at each purification stage were screened in order to shorten the process of identifying the active molecules. Chapter 2 gives a detailed account of the bioassay directed isolation and structure determination process as it was applied to the CH_2Cl_2-MeOH (1:1) extracts of the Argentinean plant *Vassobia breviflora*. This work ended up with the identification of one anticancer active compound, withaferin A.

When an active compound was identified, the mechanistic study of its activity was initiated, and in order to establish its structure-activity relationships (SAR), following efforts would be focused on either synthesizing analogues of this compound or searching for analogues from the same plant species or other related plants in the same genus or family. In Chapter 2, the synthesis of three analogues of withaferin A and the results of their anti-proliferative studies were described. In Chapter 3, 10 other withaferin A-like compounds were isolated from a related member in the Solanaceae, *Withania somnifera*.

Since the anticancer mechanistic study of withaferin A showed prominent results (Chapter 2), to continue the pre-clinical study and further clinical study and establish a completely SAR for its anticancer activity, a large quantity of withaferin A was required for these purposes. A method for large-scale isolation of withaferin A from *W. somnifera* was developed and presented in Chapter 4. The method was developed to isolate gram-scale withaferin A efficiently from a kilogram-scale of raw plant materials.

II. WITHANOLIDES FROM *VASSOBIA BREVIFLORA*

2.1 Introduction

Head and neck squamous cell carcinoma (HNSCC) represents the fifth leading cause of cancer in humans, with over 780,000 new cases diagnosed yearly worldwide.[8-10] Platinum-based therapy remains the most effective treatment option for HNSCC, but due to its significant toxicity, including ototoxicity, peripheral neuropathy, and nephrotoxicity, it may lead to up to 15% of deaths.[11] New treatment options such as molecular-targeted therapies are being explored to improve survival rates. Epidermal growth factor receptor (EGFR) targeting drugs, such as Cetuximab, have shown some advantages but limited efficacy.[12] Novel drugs discovered for this disease, especially from natural product sources, would provide a promising avenue for preclinical exploration in HNSCC.

Our program routinely screens plant extracts for anticancer activity in HNSCC in collaboration with Dr. Mark Cohen of the KU Medical Center. A library of extracts from 200 plants from Latin America was evaluated for their cytotoxic activities against four human HNSCC cell lines (JMAR, MDA 1986, UM-SCC-2 and JHU011 cells) using a MTS (3-[4,5-dimethylthiazol-2yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2*H* tetrazolium) viability assay. Further fractionation and bioassay-guided isolation of the most active plant extracts led to the isolation and identification of anti-proliferative compounds. *Vassobia breviflora* (Sendtn.) Hunz (Solanaceae) was identified as the most active of the species tested in the study.

2.2 Botany and Collection of *V. breviflora*

Vassobia, a member of the family Solanaceae, is related to *Acnistus*, *Dunalia*, and *Iochroma*, which are also genera endemic to South America.[13] *Vassobia* belongs to the subtribe *Iochrominae* and includes seven species, which are *V. atropoides, V. breviflora, V. brevifolia, V. dichotoma, V. fasciculata, V. iochromoides* and *V. lorentzii*. The plant *V. breviflora*, commonly known in Argentina as "pucancho" and "uchucho", is a widespread spiny shrub with small, purple, campanulate and glabrous flowers.[14] *V. breviflora* has been reported to have antimicrobial activity.[15]

Aerial parts of *V. breviflora* were collected as part of an NIH International Cooperative Biodiversity Groups Program. The plant biomass was collected by R. Fortunato and A. Cabral along Highway RN 86, 2 km north east of Primavera, Department Patino, Province of Formosa, Argentina (lat. 25°14'0"S, long. 57°57'0"W) on November 25, 1999. A voucher specimen (No. ARP 612) identified by R. Fortunato was deposited in the Herbarium (BAB) of the Institute of Biological Resources, National Institute of Agricultural Technology (INTA), Castelar, Buenos Aires, Argentina. Intellectual Property Rights Agreements for plant collections and interdisciplinary research have been executed between the collaborating institutions in the US and Argentina.

2.3 Systematics and Phytochemistry of the Solanaceae

The large family Solanaceae, the nightshade family, contains 102 genera and nearly 2500 species distributed worldwide.[16] Based on biological effects and since ancient times, humans have used plants of the Solanaceae for the prevention and treatment of diseases. For instance, *Mandragora officinarum* was valued in ancient Greece and Rome for its intoxicating and narcotic properties and was used as a surgical anesthetic.[17] Furthermore, the common vegetables potatoes, peppers, eggplants and tomatoes in this family make it economically important as the basis of human food. Solanaceae and Convolvulaceae are sister families in the order of Solanales sharing similar phylogeny, morphologic-anatomical properties, phytogeographic distribution, and secondary metabolism.

The Solanaceae family contributes high-valued natural products used in traditional medicine, pharmacology and drug therapy. Alkaloids, phenolics, flavonoids, lignans, sterols, carotenoids, capsaicinoids, steroidal saponins, steroidal alkaloids, withanolides, phytosterols and petuniasteroids are examples of specific secondary metabolites present in this family.

Withanolides, a significant group of compounds in the Solanaceae, are steroidal lactones built on an ergostane skeleton of 28 carbons. The first withanolide, "withaferin A", was discovered as a constituent of the leaves of *Withania somnifera*, also called Indian Ginseng, by Lavie et al. in 1965.[18] To date, over 300 withanolides have been isolated, most of them from genera belonging to the Solanaceae.[19] Besides *Withania,* the genera *Acnistus, Datura, Deprea, Exodeconus, Iochroma, Jaborosa, Physalis, Salpichroa* and *Vassobia* are reported to contain withanolide-like compounds.

2.4 Bioassay Guided Fractionation

In order to determine the source of the anticancer activity in *V. breviflora*, a bioactivity guided fractionation was employed. This method enabled the isolation of bioactive compounds without having to examine every component of an extract or fraction. At each stage of purification, the fractions obtained were screened for their anticancer activities in JMAR, MDA 1986, UM-SCC-2 and JHU011 human HNSCC cells using the MTS assay.

Dried and ground aerial parts of *V. breviflora* (100 g) were extracted at room temperature with CH_2Cl_2-MeOH (1:1) three times to afford 9.8 g of a brown crude extract. The crude extract exhibited activity against MDA1986, JMAR, UM-SCC-2 and JHU011 cancer cell lines using MTS test with the half-maximum inhibitory concentration (IC_{50}) values of 1.1, 7.3, 0.94 and 1.1 μg/mL respectively. The crude extract was dissolved in 500 mL of MeOH-H_2O (1:9) and then placed in a separatory funnel to which 500 mL of hexane were added in order to remove the non-polar lipophilic organics such as fatty acids. The hexane layer was subjected to two additional liquid-liquid partitions. The hexane layers were combined and concentrated under reduced pressure to obtain a total of 0.79 g of a crude hexane extract. The water layers were each further placed in the separatory funnel to which 500 mL of EtOAc were added in order to extract organic compounds of mid-polarity. The extraction process was repeated twice and the organic layers were combined and concentrated under reduced pressure to yield a total of 2.65 g of a crude EtOAc extract. Subsequently, the water layers were each extracted with BuOH three times (500 mL × 3) to afford a total of 2.63 g of a crude BuOH extract, which contained most of the

polar organics. After all the described extraction procedures were completed, the aqueous layers were discarded. Among the three extracts, the EtOAc extract showed the highest activity against MDA1986, JMAR, UM-SCC-2 and JHU011 cancer cell lines using MTS test with the half-maximum inhibitory concentration (IC_{50}) values of 1.2, 6.7, 0.87 and 1.2 μg/mL respectively. In order to narrow down the search for the active constituents in the most active sample, we concentrated our isolation and characterization studies to the EtOAc extract.

The EtOAc extract was subjected to a silica gel column chromatography (32-60 μm, 5 × 60 cm) and eluted with gradient mixtures of CH_2Cl_2-MeOH (100:0, 98:2, 95:5, 93:7, 90:10, 80:20, 50:50 and 0:100), in order of increasing polarity. A total of 182 fractions were collected, and after pooling similar fractions based on TLC profiles, fractions 49-54 (830.3 mg; CH_2Cl_2-MeOH 93:7) were combined and confirmed by MTS assay as the most active fractions. These fractions were further purified by normal-phase SPE (20 g; 60 ml) using a step gradient of CH_2Cl_2-MeOH mixtures (90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, 50:50, and 0:100). Among the nine collected fractions (**Table 2.1**), fraction 5 (CH_2Cl_2-MeOH 70:30) was the most active one by displaying anti-proliferative activity against MDA1986, JMAR, UM-SCC-2 and JHU011 cancer cell lines with IC_{50} values of 0.9, 1.0, 1.0 and 1.1 μg/ml. When examined by TLC, fraction 5 showed one major red spot (compound **1**) when sprayed with $CHCl_3$-Hexane-EtOAC-MeOH (72:40:18:8). After crystallization using 20 mL of $CHCl_3$-MeOH (1:1), 50 mg of compound **1** were obtained. Additionally, 20 mg of Fraction 3 (CH_2Cl_2-MeOH 80:20) were obtained and its TLC showed the presence of one purple spot (compound **2**).

Table 2.1. Fractionation of Fractions 49-54 from EtOAc extract of *V. breviflora*.

Fractions	Wt. (in mg)	%Yield[a]
1	21	2.5
2	86	10.3
3	20	2.5
4	32	3.9
5	78	9.4
6	121	14.6
7	148	17.8
8	21	2.5
9	145	17.4

[a]As a percentage of Fractions 49-54 (830.3 mg) from EtOAc extract of *V. breviflora*.

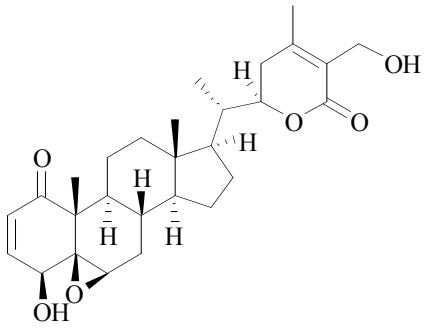
2.5 Structure Elucidation and Identification

Compound **1** (**Figure 2.1**) was isolated as colorless block-shape crystals. Its HRMS showed an $[M+H]^+$ ion at m/z 471.2730 corresponding to the molecular formula $C_{28}H_{39}O_6$. The IR spectrum suggested the presence of hydroxyl (3421 cm^{-1}) and carbonyl (1678 cm^{-1}) groups. The ^{13}C and DEPT spectra indicated the presence of four methyls, seven methylenes (including one oxygenated at δ 57.0), ten methines (including two olefins at δ 142.5 and δ 132.3), and seven quaternary carbons (**Table 2.2**). Of the four methyls, three were identified as tertiary methyls base on HSQC and ^1H spectra. In HMBC spectra, the tertiary methyl protons at δ 0.71 had strong correlations with carbon at δ 39.2, δ 42.5, δ 51.8 and δ 56.0; the tertiary methyl protons at δ 1.42 had strong correlations with carbon at δ 44.0, δ 47.8, δ 63.9 and δ 202.3. Based on HSQC and HMBC information, a steroidal skeleton was assigned to compound **1**. The tertiary methyl protons on the side chain at δ 2.03 were correlated to one methylene carbon at δ 29.8 and two olefins at δ 125.6 and δ 153.5 in HMBC, indicating the presence of an unsaturated lactone side chain. Therefore, a withanolide structure was identified based on 1D and 2D NMR spectra (**Figure 2.2**). In addition, compound **1** co-chromatography with an authentic withaferin A sample purchased from Chromadex (Irvine, CA) confirmed its structural assignment. Furthermore, the X-ray structure (**Figure 2.3**) of compound **1** was also calculated as withaferin A.

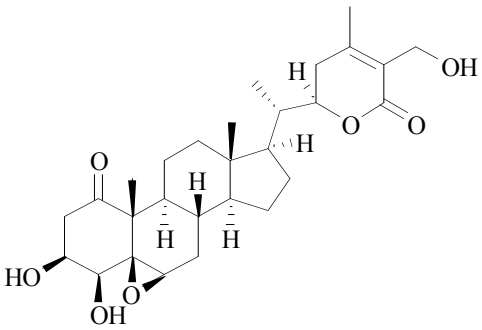
Compound **2** (**Figure 2.1**) was determined to be a close analog of compound **1** from the 1D and 2D NMR spectra. Instead of an α, β-unsaturated ketone, the double bond on ring A (C-2, δ132.3 and C-3, δ142.5 in compound **1**) was reduced and an additional hydroxyl group was attached to

carbon three in **2**(C-2, δ43.9 and C-3, δ69.6 in compound **2**). The rest of the structure was determined to be the same as in withaferin A based on NMR data (**Figure 2.4**). Compound **2** was identified as viscosalactone B by comparison of its NMR data with those reported in the literature.[20]

Figure 2.1. Withaferin A (**1**) and Viscosalactone B (**2**) from *V. breviflora.*



1



2

Table 2.2. ^{13}C NMR data for withaferin A (**1**) and viscosalactone B (**2**).

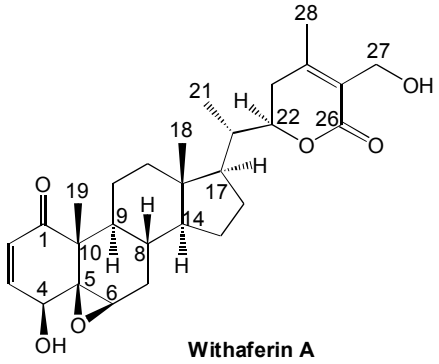
Positions	***1***[a]	***2***[b]
1	202.3	213.0
2	132.3	43.9
3	142.5	69.6
4	69.8	78.9
5	63.9	65.7
6	61.7	59.9
7	29.8	31.1
8	31.1	32.6
9	44.0	50.0
10	47.8	51.9
11	21.8	22.5
12	39.2	28.3
13	42.5	44.3
14	56.0	56.5
15	24.2	25.6
16	27.2	28.6
17	51.8	53.2
18	11.6	12.1
19	17.0	15.3
20	38.7	40.5
21	13.3	13.8
22	78.7	80.2
23	29.8	30.8
24	153.5	158.0
25	125.6	126.5
26	167.0	168.6
27	57.0	57.4
28	20.0	20.4

[a] ^{13}C NMR data for withaferin A (**1**) recorded in $CDCl_3$ at 125 MHz.

[b] ^{13}C NMR data for viscosalactone B (**2**) recorded in CD_3OD at 125 MHz.

Figure 2.2. 1D and 2D NMR spectra of withaferin A (**1**).

A. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of withaferin A (**1**).



Withaferin A

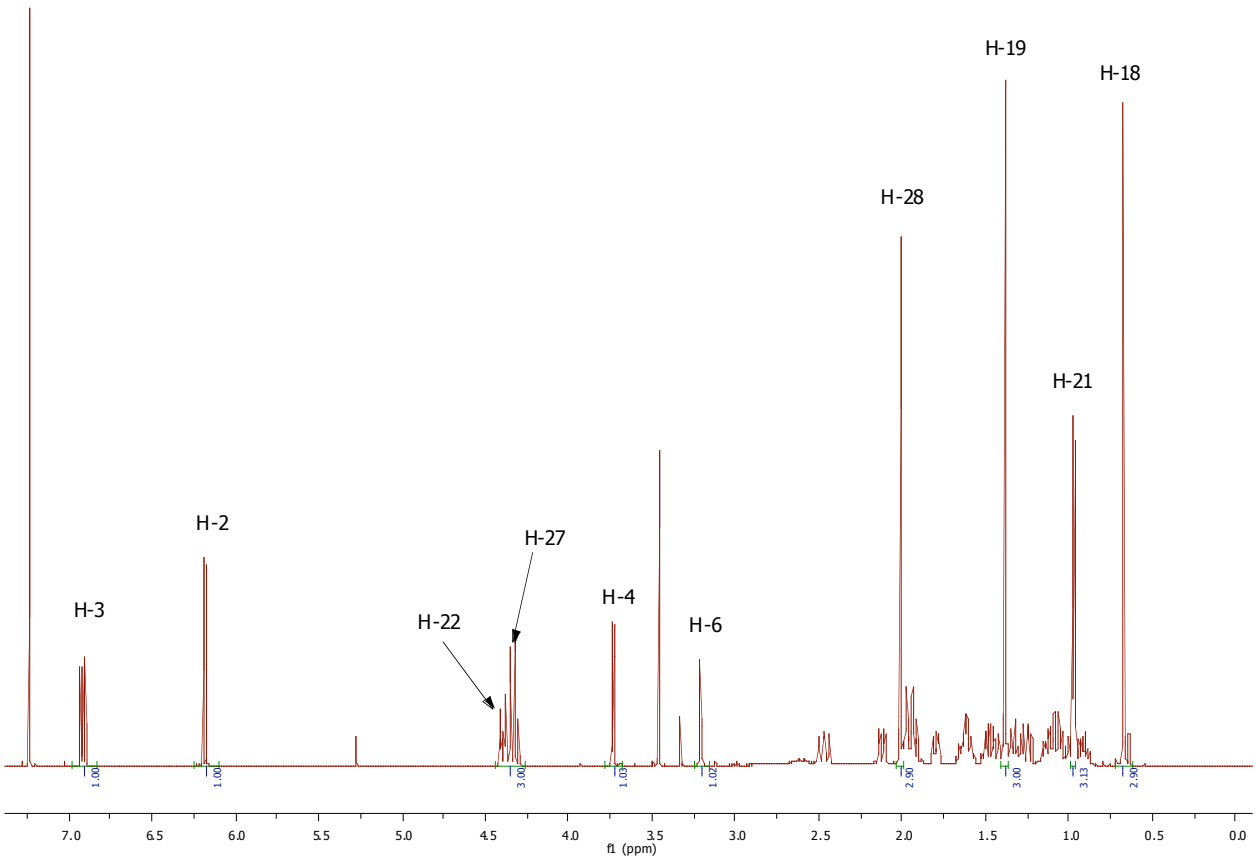


Figure 2.2. 1D and 2D NMR spectra of withaferin A (**1**) – (cont.)

B. COSY spectrum of withaferin A (**1**).

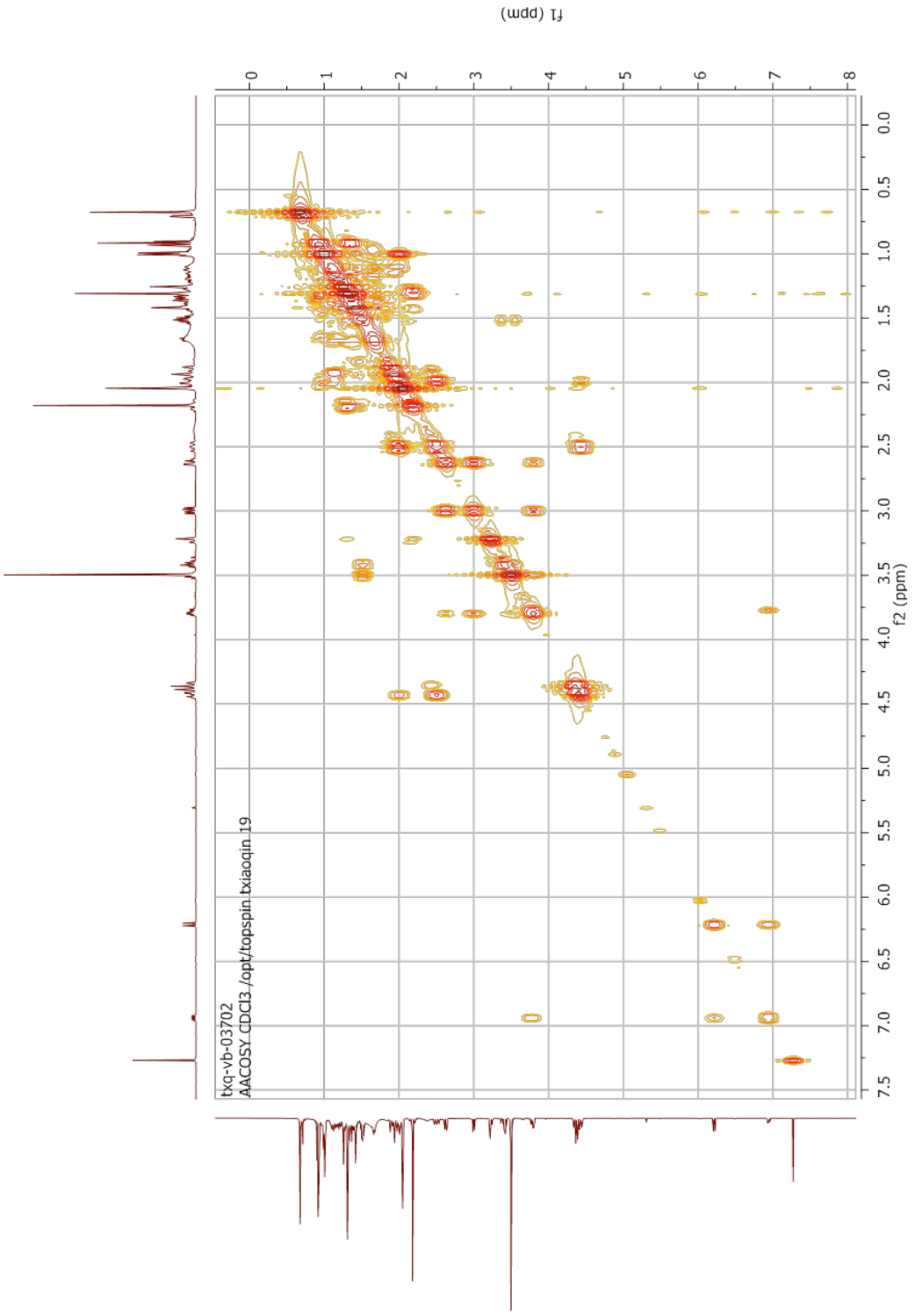


Figure 2.2. 1D and 2D NMR spectra of withaferin A (**1**) - (cont.)

C. HSQC spectrum of withaferin A (**1**).

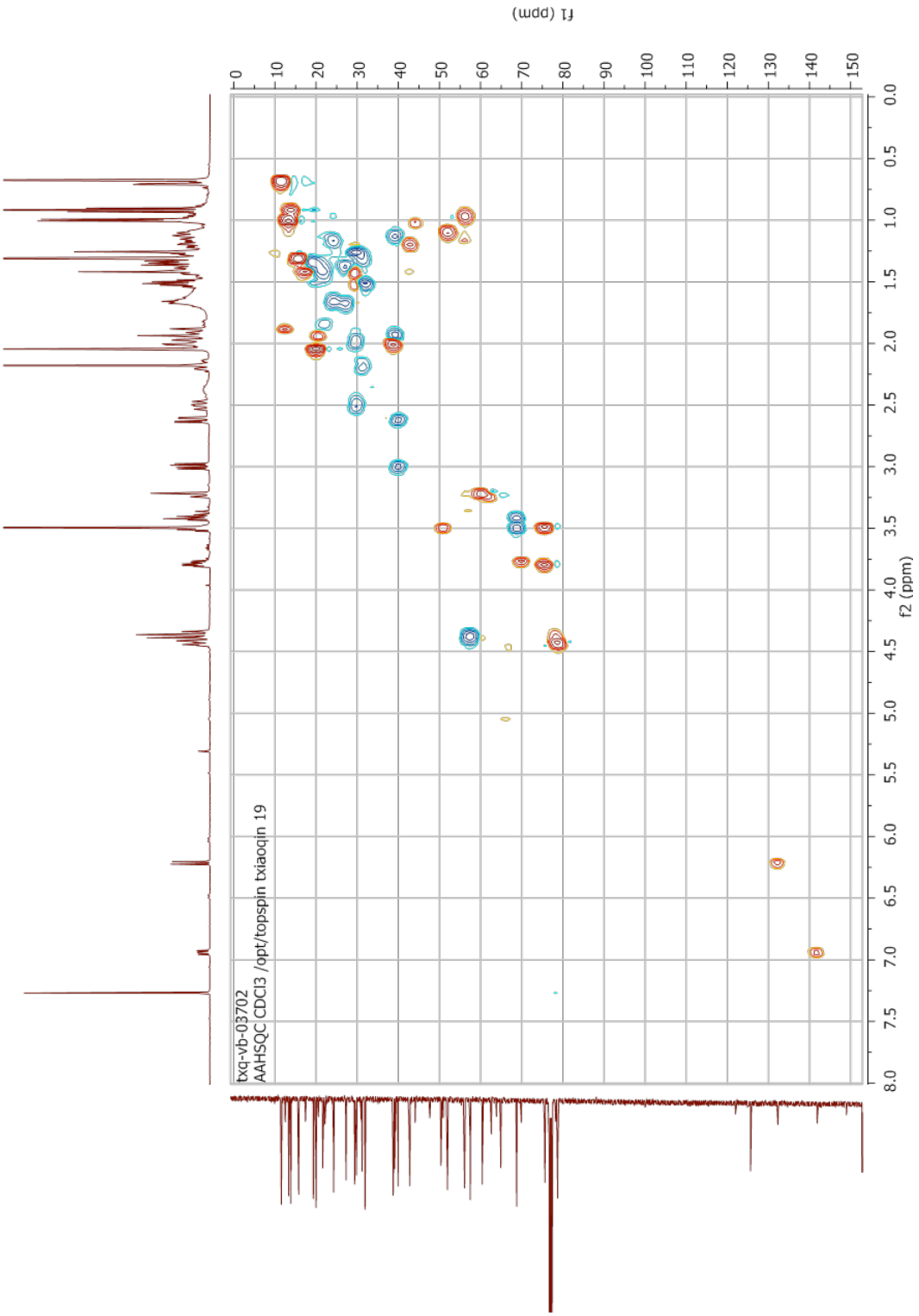


Figure 2.2. 1D and 2D NMR spectra of withaferin A (**1**) **- (**cont.)

D. HMBC spectrum of withaferin A (**1**)**.**

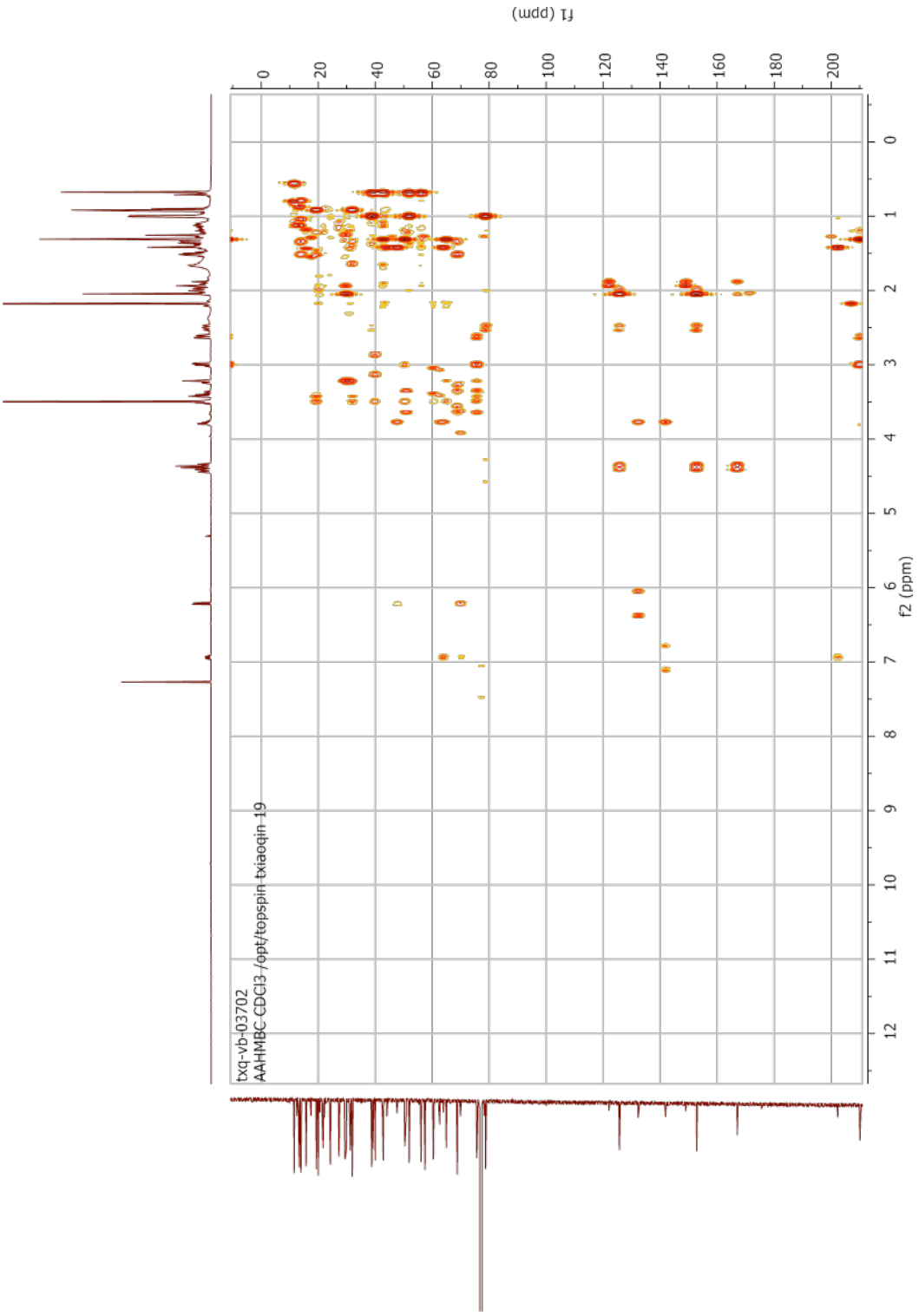


Figure 2.3. ORTEP view of withaferin A (**1**) from X-ray diffraction data.

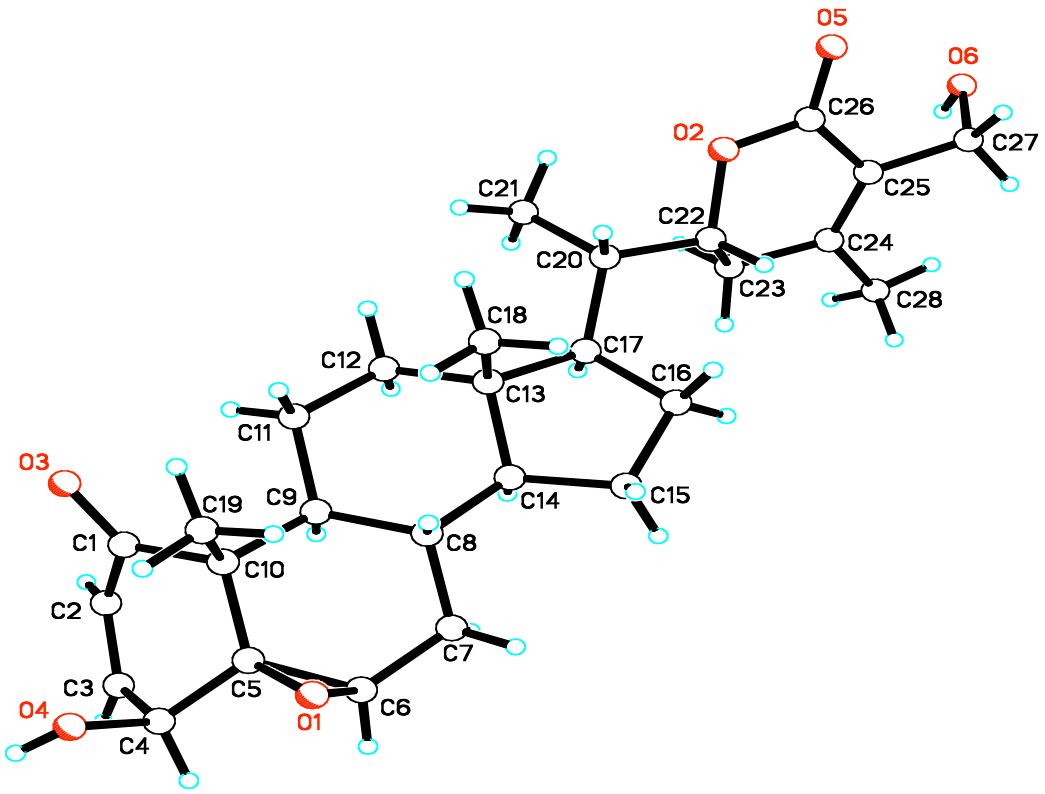
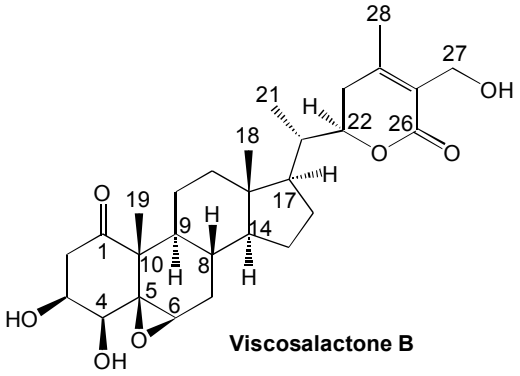
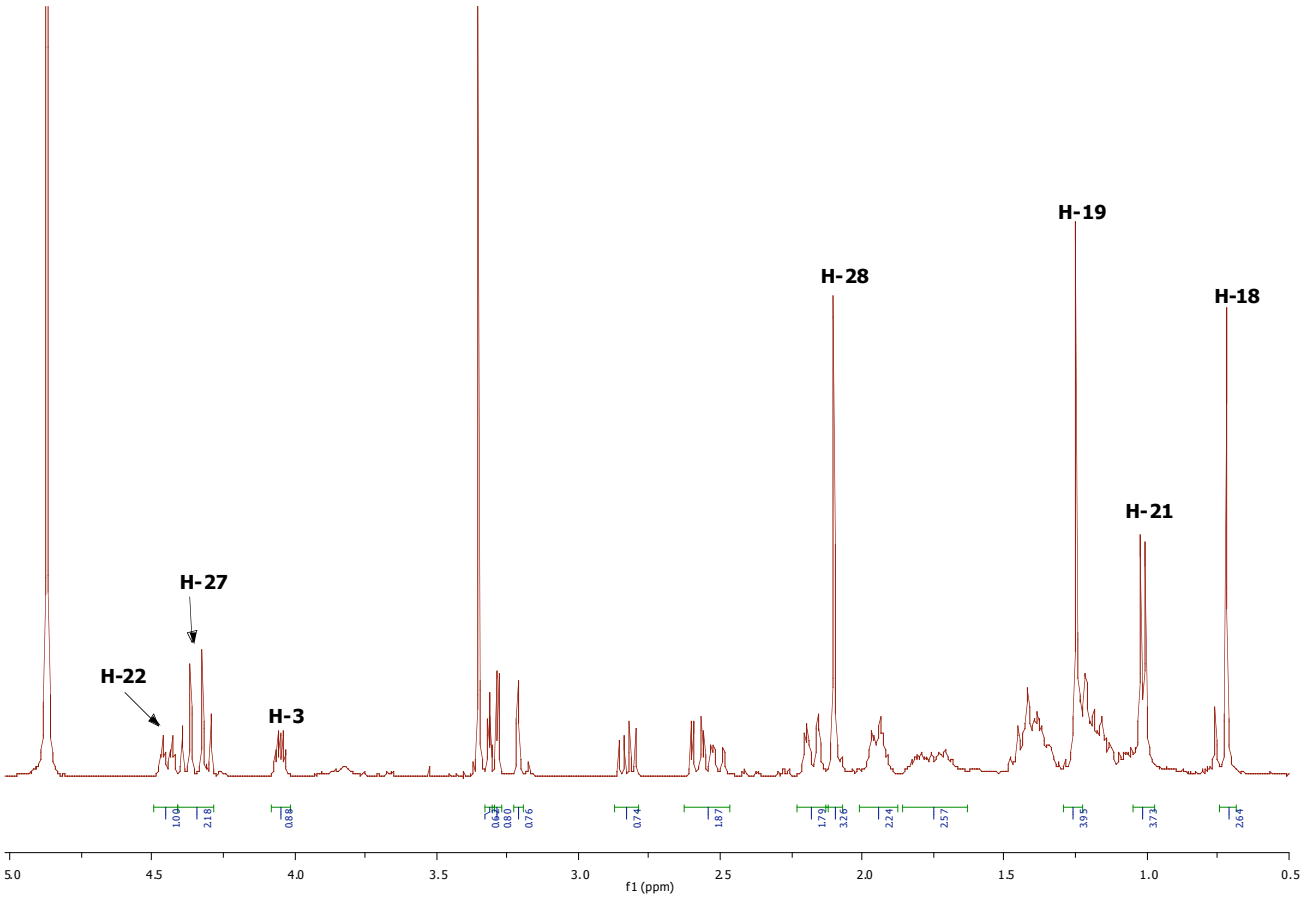


Figure 2.4. ^{1}H NMR spectrum (500 MHz, CD_3OD) of viscosalactone B (**2**).



Viscosalactone B



2.6 Anti-proliferative Activity

The anticancer activities of all isolates from *V. breviflora* were determined against a variety of cancer cell lines using a MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium] assay. Compound **1** reduced cell viability of HNSCC cell lines and inhibited their growth in a concentration dependent manner. The IC_{50} values in UM-SCC-2, MDA1986, JMAR and JHU-011 cells were 0.5, 0.8, 2.0 and 2.2 μM, respectively. The results showed that these four cell lines were very sensitive to the treatment with compound **1** while the most sensitive one was the UM-SCC-2 cell line. Compound **1** also showed anti-proliferative activity against melanoma cell lines B16F10, NPA, DRO and SKMEL28 with the IC_{50} values 0.7, 1.3, 1.4 and 3.3 μM, respectively. Compound **2** was less active than compound **1** in our assay against HNSCC cell lines, showing no activity with the concentration of 1 μg/mL.

2.7 Anti-proliferative Mechanistic Study

In order to investigate a possible mechanism for the anti-proliferative effect of compound **1**, a series of experiments to evaluate cell-cycle arrest and the apoptotic pathway were performed. As shown in **Figure 2.5**, treatment of MDA1986, JMAR, UM-SCC-2, and JHU011 HNSCC cells with 3.0 μM of compound **1** for 24 h induced a shift of cells from G_0/G_1 arrest to a significant increase in the percentage of cells in the G_2/M phase, from 27% to 42% in JMAR cells (**Figure 2.5**A) and from 26% to 49% in MDA1986 cells (**Figure 2.5**B; $p < 0.01$). The G_2/M phase shift for UM-SCC-2 cells was from 27% to 36% (**Figure 2.5**C) and for JHU011 cells, 25% to 34% (**Figure 2.5**D, $p < 0.01$). Compound **1** additionally induced S phase arrest in JMAR, UM-SCC-2

and JHU011 cells. Treatment with compound **1** also led to an increase in sub-G_0 levels in both JMAR (from 2% to 12%; p <0.01) and MDA1986 cells (from 7% to 8%; not significant), suggesting that JMAR cells underwent DNA fragmentation, one of the early biochemical events leading to apoptosis.

In order to confirm the apoptotic action induced by compound **1**, cells were treated with 5.0 μM of compound **1** for 24 h and stained with annexin V/propidium iodide (PI). As shown in **Figure 2.6**, treatment of HNSCC cells with compound **1** increased the annexin V-positive cells. Staining only with Annexin V signified the early apoptotic events whereas dual staining with annexin V and PI indicated the late phase of apoptosis. Treatment of JHU011 cells with compound **1** induced early apoptotic events (p <0.01) significantly from basal levels of 4.8% to 14.8%. There was a slight increase in the late phase of apoptosis induced by compound **1** in JHU011 cells, with very small staining only with PI (necrosis) observed in JHU011 cells treated with compound **1**. Treatment of JMAR and UM-SCC-2 cells with compound **1** induced significant increases in early and late apoptosis as well as necrosis (p <0.01). MDA1986 cells seemed less sensitive to treatment with compound **1**, since withaferin A did not increase annexin V/PI staining herein.

To confirm the annexin V studies on HNSCC cells, caspase 3 activation was investigated in HNSCC (**Figure 2.7**). Treatment of JHU011 cells induced activation of the caspase 3 enzyme, as signified by decreased procaspase 3 levels. In addition, the caspase 3 substrate PARP was cleaved upon treatment with **1,** in a concentration-dependent manner (**Figure 2.7**). Treatment with compound **1** at 1.0 μM increased PARP cleavage in JHU011 cells. JMAR cells treated with compound **1** also showed similar decreases in procaspase 3 levels and cleavage of PARP protein,

in a concentration-dependent fashion. Similar results were also observed when UM-SCC-2 cells were treated with compound **1**. Treatment of MDA1986 cells with withaferin A increased caspase 3 activation and PARP cleavage. However, in line with the annexin V/PI data, a slightly higher concentration of compound **1** was required to induce caspase 3 activation in MDA1986 cells (**Figure 2.7**). These results indicate that compound **1** induced cell-cycle arrest at the G_2/M phase and apoptosis was confirmed by positive annexin V/PI staining as well as caspase 3 activation in the HNSCC cell lines investigated. The G_2/M cell cycle arrest is a typical marker of apoptosis, and defects in the G_2/M arrest check point may allow a damaged cell to enter mitosis and undergo apoptosis.[21] At this point, it is not clear whether G_2/M cell cycle arrest provides conditions inducive of caspase 3 activation.

The effect of withaferin A (**1**) was examined on major prosurvival signaling pathways in HNSCC cells including PI3 kinase/Akt and ERK1/2 MAP kinase. These data showed that compound **1** reduced phosphorylation (ser473) as well as total Akt levels in all HNSCC cells in a concentration-dependent manner (**Figure 2.8**). Treatment of JHU011, JMAR, and UM-SCC-2 cells with as low as 100-250 nM concentrations of compound **1** reduced Akt phosphorylation. It is interesting to note that a higher concentration of compound **1** was required to inhibit Akt activity in MDA1986 cells (2.5 μM). In addition to the effect of compound **1** on the reduction of phosphorylated Akt, the total Akt levels were decreased significantly in all cell lines examined (**Figure 2.8**). These data show that compound **1** is a strong Akt inhibitor in HNSCC cells. The effect of compound **1** was examined on ERK1/2 activation in HNSCC cells. Withaferin A increased phosphorylation of ERK1/2 MAP kinase in JHU011, JMAR, and MDA1986 cells. Only at higher concentrations did compound **1** inhibit activation of ERK1/2 in UMSCC-2 cells.

Withaferin A had no effect on total ERK1/2 levels. All the anti-proliferative activity and mechanism studies have been done by Dr. Mark Cohen and his group, and the collaborative results were published in 2010.[22]

Figure 2.5. Detection of S and G_2/M cell cycle arrest in withaferin A (**1**) treated cells by flow cytometry. JMAR (A) MDA1986 (B), UM-SCC-2 (C), and JHU011 (D) cells were treated with DMSO or 3.0 µM **1**. Cells treated with vehicle (DMSO) were primarily at the G_0/G_1 phase, while cells treated with compound **1** showed shifts from the G_0/G_1 to the S and G_2/M phases.

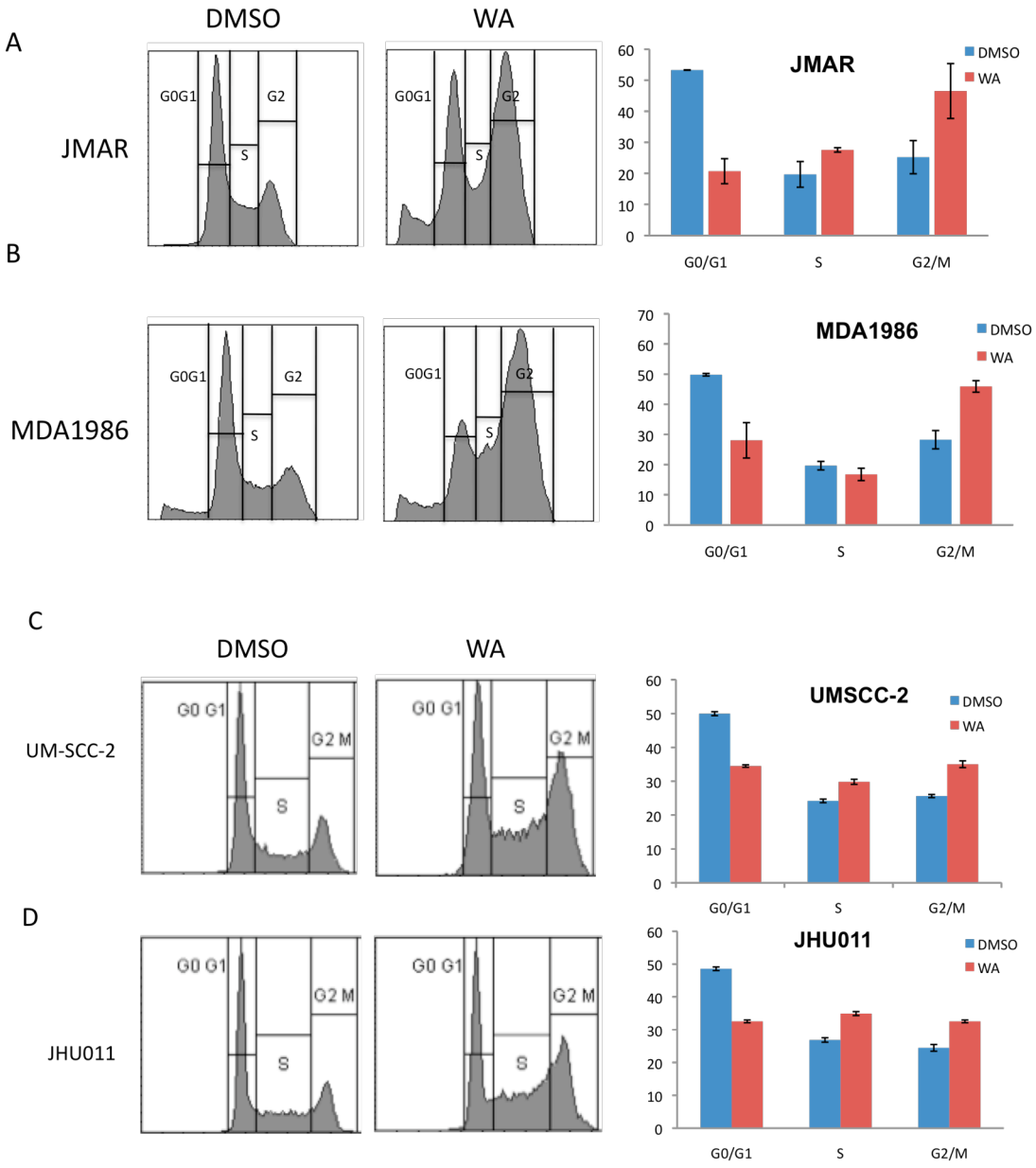


Figure 2.6. Withaferin A (**1**) induced apoptosis in head and neck squamous cell carcinoma (HNSCC) cells. HNSCC cells were treated with 5.0 μM of **1** for 24 h and then harvested and stained with annexin V and propidium iodide followed by analysis by flow cytometry. All experiments were performed in triplicate. Treatment with **1** exhibited an increase in annexin V and PI staining that indicated early as well as late apoptotic events. JMAR cells treated with **1** showed significant necrosis.

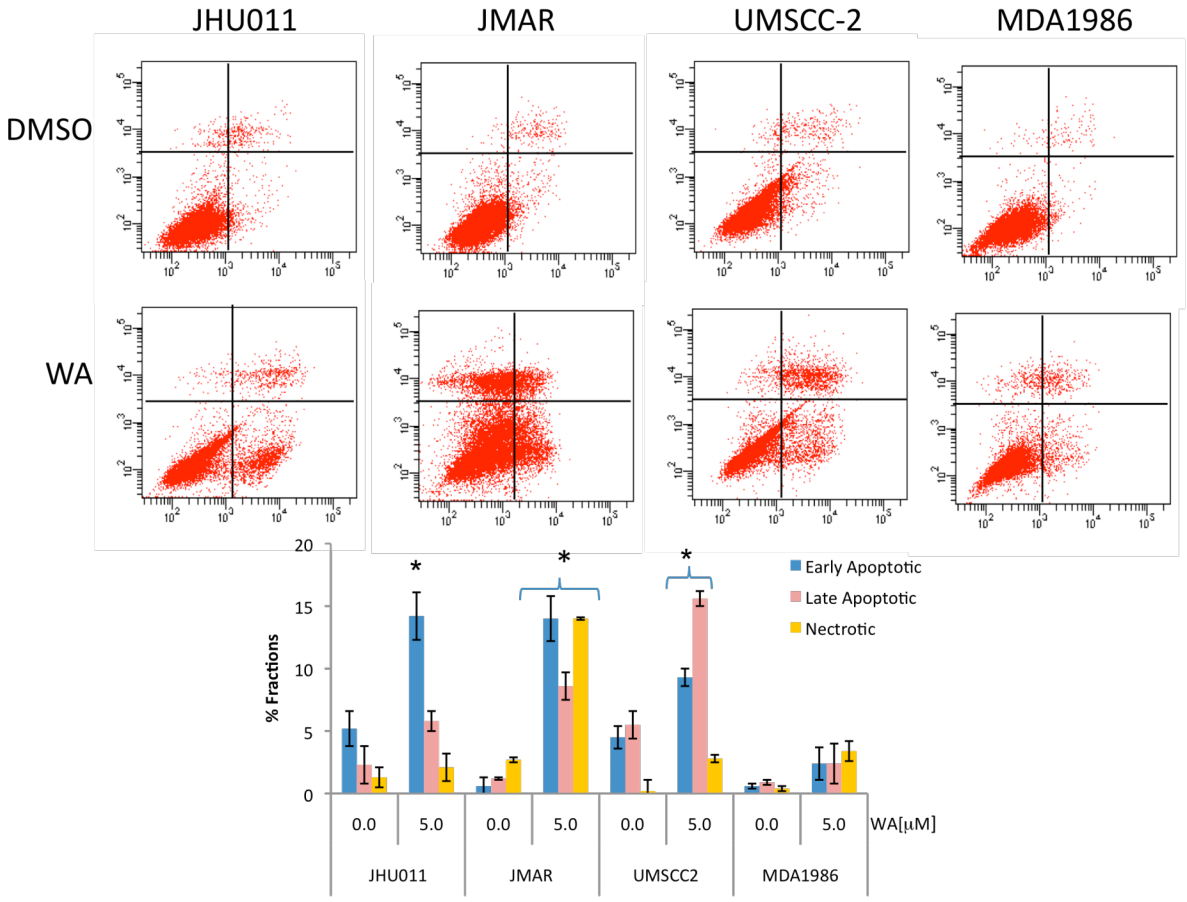


Figure 2.7. Caspase 3 was activated in head and neck squamous cell carcinoma cells treated with withaferin A (**1**). HNSCC cells were treated with different concentrations of **1** for 24 h. Levels of pro-caspase 3 (inactive form) were examined with the antibody against uncleaved caspase 3. In addition, activation of caspase 3 was examined with levels of the uncleaved and cleaved caspase 3 substrate, PARP. Compound **1** induced caspase 3 activation in all HNSCC cell lines in a concentration-dependent manner.

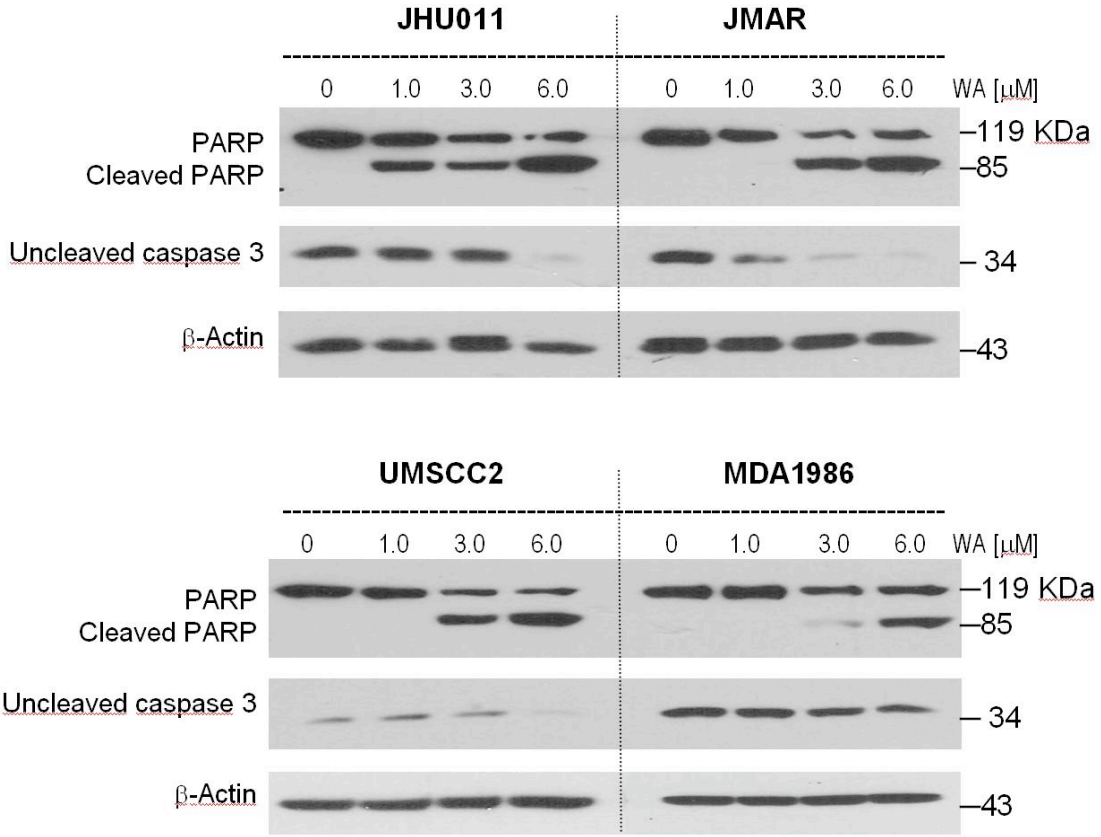
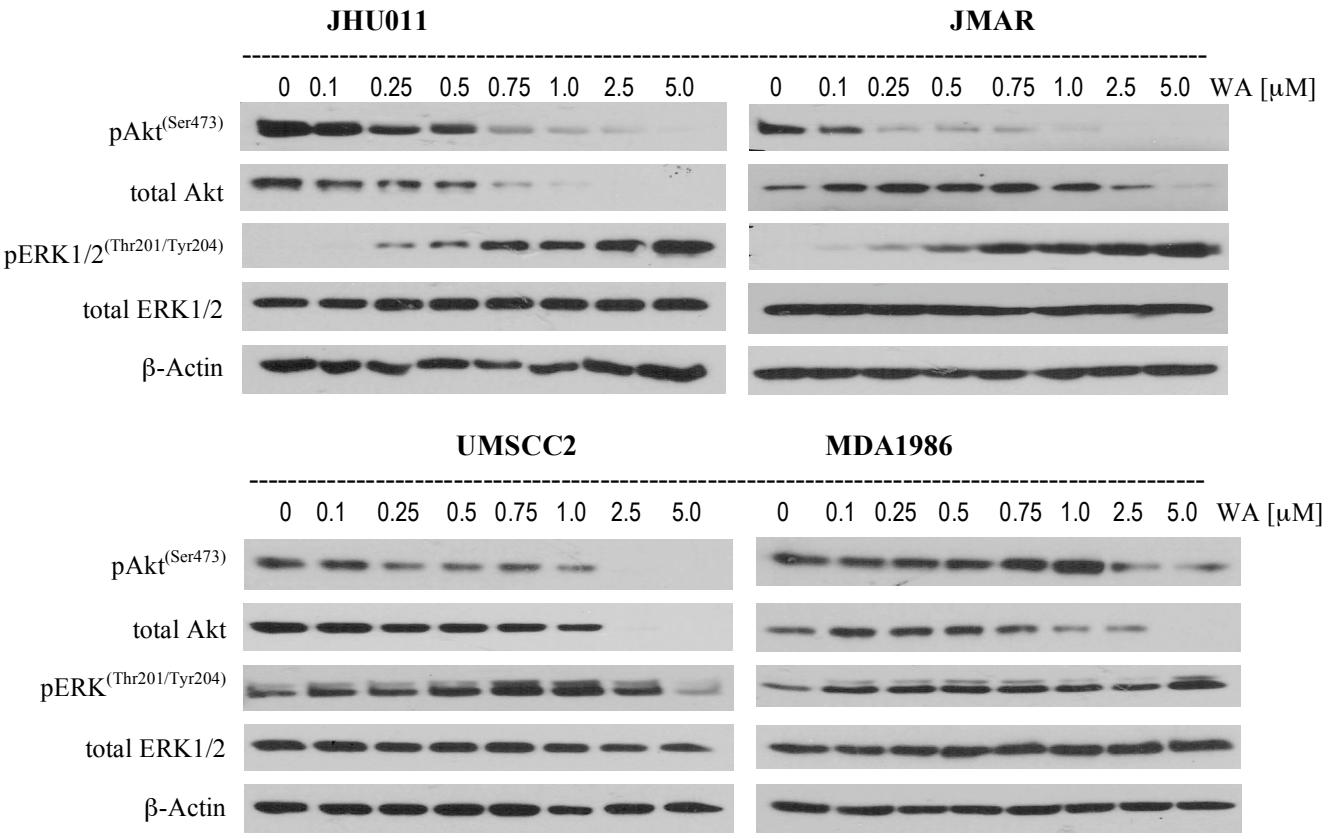


Figure 2.8. Compound **1** modulated prosurvival signal transduction pathway. HNSCC cells were treated with increasing concentrations of **1** for 24 h. Total levels of phospho-Akt, total Akt, phospho-ERK and total ERK2 were determined by Western blotting. Antibody against β-actin was used as a protein loading control. Compound **1** reduced activation of Akt as well as total cellular Akt, while ERK1/2 was activated in JHU011, JMAR, and MDA1986 cells while ERK1/2 activity decreased in UM-SCC-2 cells at a high concentration of **1**. Total ERK levels remained unchanged with compound **1** treatment.



2.8 Synthesis of Withaferin A Analogues

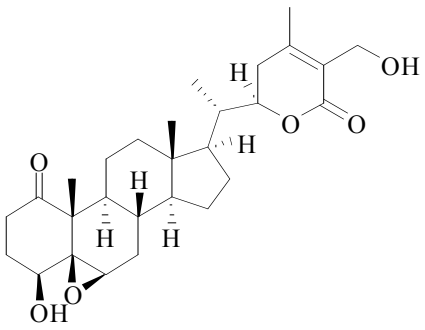
In order to investigate a possible SAR for the antiproliferative activity of withaferin A, three analogues were semi-synthesized from withaferin A. Compounds **1_a** and **1_b** (**Figure 2.9**) were prepared by step hydrogenation of withaferin A.[18] Withaferin A (10 mg) in EtOAc (3.2 mL) was hydrogenated over 10% palladium on charcoal (1 mg) at room temperature and atmospheric pressure for 3 h. The reaction solution was filtered and washed with 100 ml of MeOH, and then concentrated under reduced pressure to obtain a total of 9.5 mg of product. The TLC of the reaction product showed two major spots, which were pink (Rf = 0.3, compound **1_a**) and orange (Rf = 0.5, compound **1_b**) after spraying with vanillin-sulfuric acid reagent. To separate both compounds, this product was further purified by semi-prep HPLC eluting with CH_3CN-H_2O (50:50). Compound **1_a** (1.2 mg) was collected at the retention time of 17 min and compound **1_b** (7.8 mg) was at 28 min. Compound **1_b** was recrystallized using $CHCl_3$ and EtOAc (2:1) to afford light yellow parallelepiped-shaped crystals.

Compound **1_c** (**Figure 2.9**) was prepared by acetylation of withaferin A.[23] Withaferin A (5 mg) was dissolved in dry pyridine (350 μL) and mixed with an excess of acetic anhydride (1 mL). The reaction mixture was refluxed for 1 h (about 60 ºC) and then poured into ice-cold water. A white crystalline precipitate was formed in the mixture, which was then extracted with CHCl3 (50 mL) using a separatory funnel. The organic layer was concentrated under reduced pressure to afford 4.9 mg of product. This reaction product was further purified by a silica gel column (5 g, 12~26 μm, Sorbent Technologies), which was eluted with EtOAc-hexane (1:1), to obtain 4.3 mg of compound **1_c**.

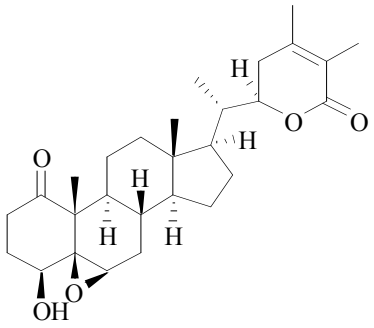
By comparison with the reported data,[24] compound **1_a** was identified as a 2,3-dihydroxy derivative of withaferin A (**1**). The obvious difference between **1** and **1_a** was that the double bond between C-2 (δ 132.3) and C-3 (δ 142.5) in withaferin A was reduced to a single bond C-2 (δ 31.8) and C-3 (δ 26.5) (**Table 2.3**) in compound **1_a** (**Figure 2.10**). The structure of **1_b** was confirmed by proton NMR (**Figure 2.11**) and through single-crystal X-ray diffraction experiments (**Figure 2.13**) as 2,3-dihydro-27-deoxywithaferin A. Compound **1_c** was confirmed as diacetylwithaferin A, which displayed two additional pairs of a carbonyl group and a methyl group compared to withaferin A on ^{13}C NMR (DEPT) spectra (**Figure 2.12** and **Table 2.3**). 1D and 2D NMR spectra and X-ray structure of compound **1** refer to **Figure 2.2** described in section 2.5.

The IC_{50} values of compound **1_c** against cancer cell lines MDA1986 and JMAR were 0.95 and 0.98 μM, which were similar to those obtained for withaferin A. Compound **1_c** was not active at 20 μM against cancer cell lines JHU011 and UMSCC2. Compound **1_a** and **1_b** did not show any activity at 20 μM against all four HNSCC cancer cell lines.

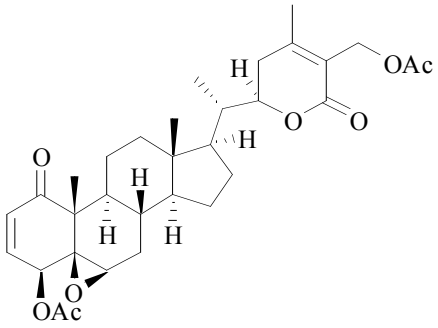
Figure 2.9. 2,3-Dihydrowithaferin A (**1$_a$**), 2,3-dihydro-27-deoxywithaferin A (**1$_b$**) and diacetylwithaferin A (**1$_c$**) synthesized from withaferin A.



1$_a$



1$_b$



1$_c$

Figure 2.10. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of 2,3-dihydrowithaferin A (**1_a**).

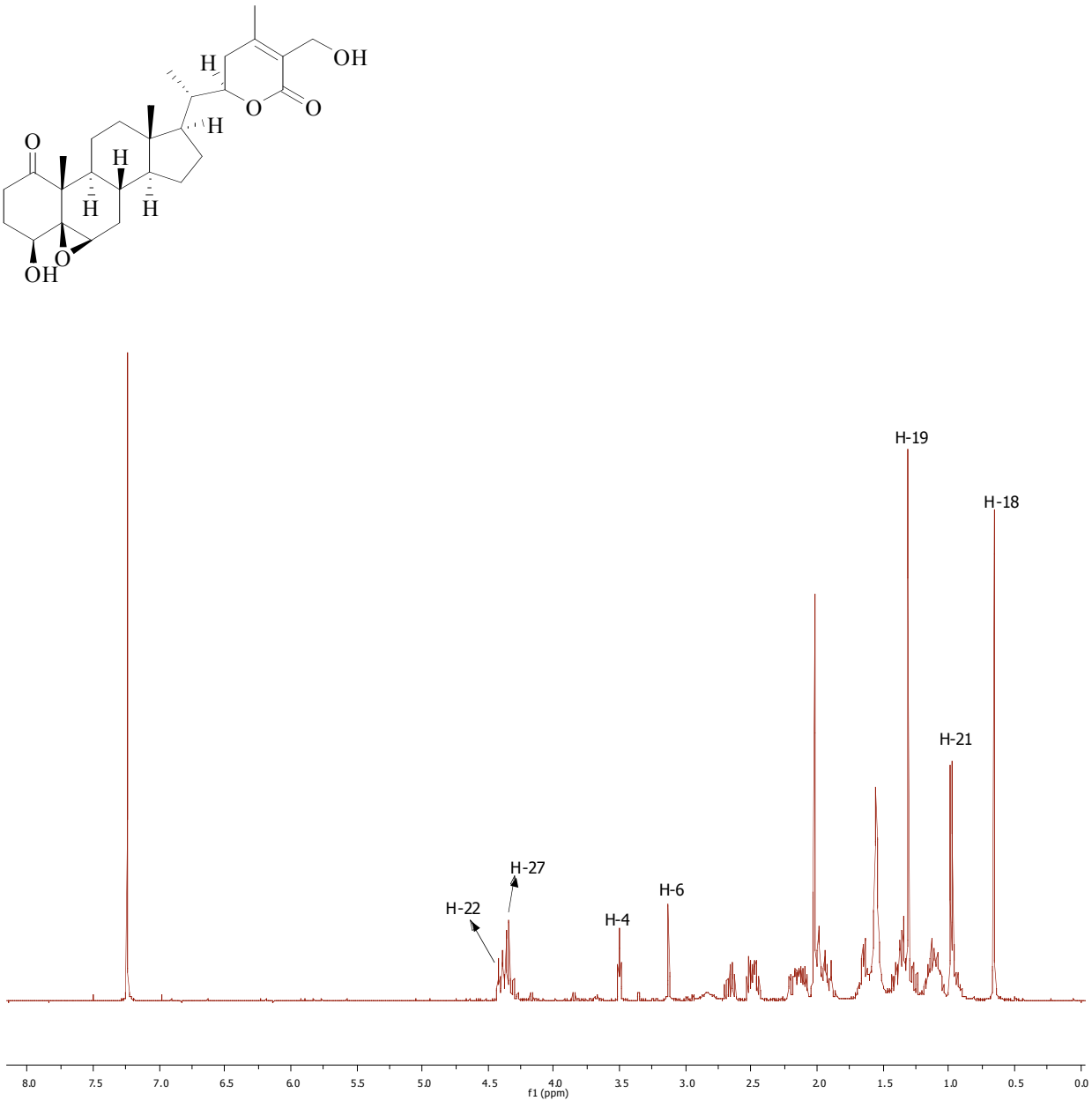


Figure 2.11. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of 2,3-dihydro-27-deoxywithaferin A (**1_b**).

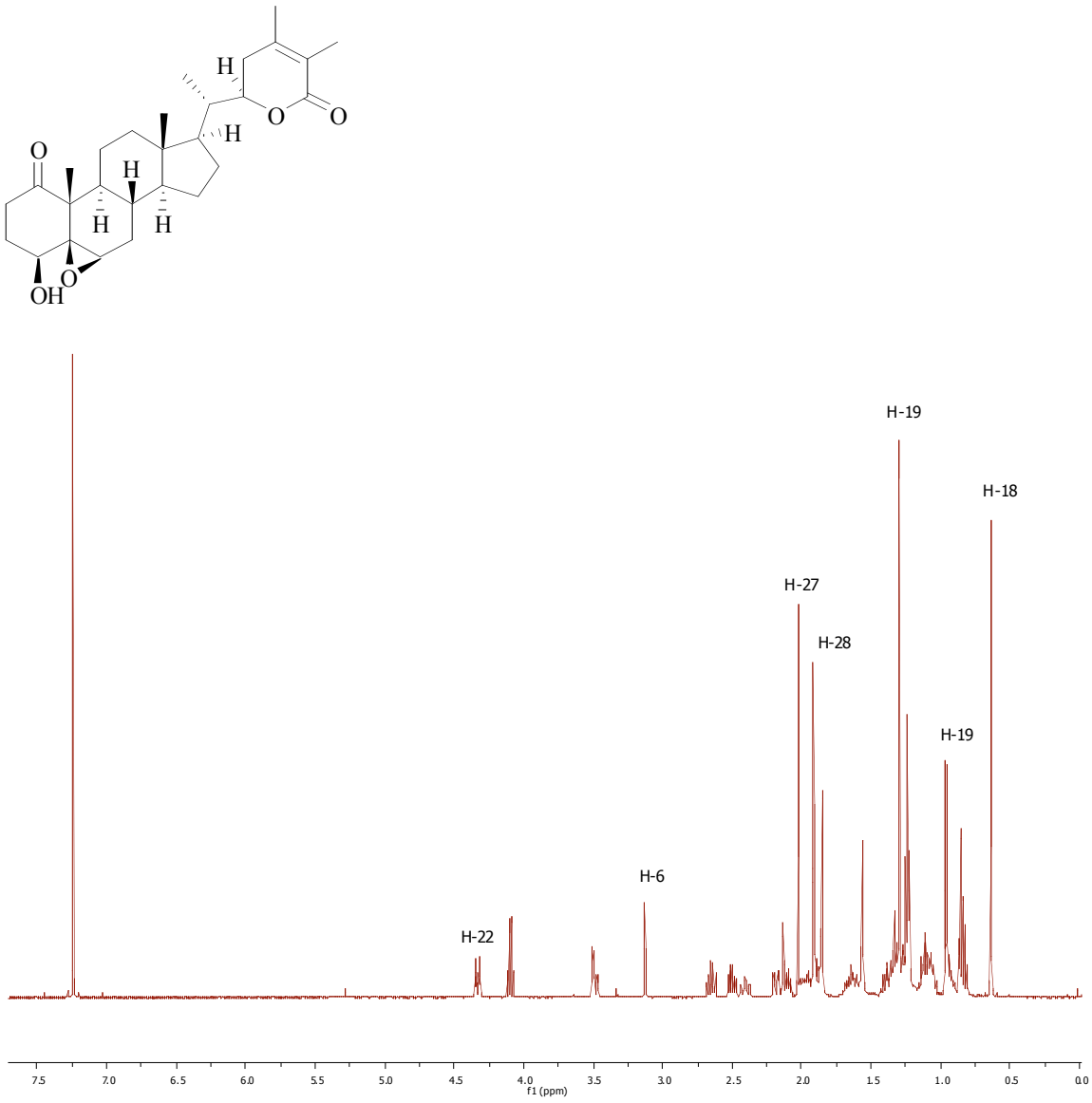


Figure 2.12. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of diacetylwithaferin A (**1c**).

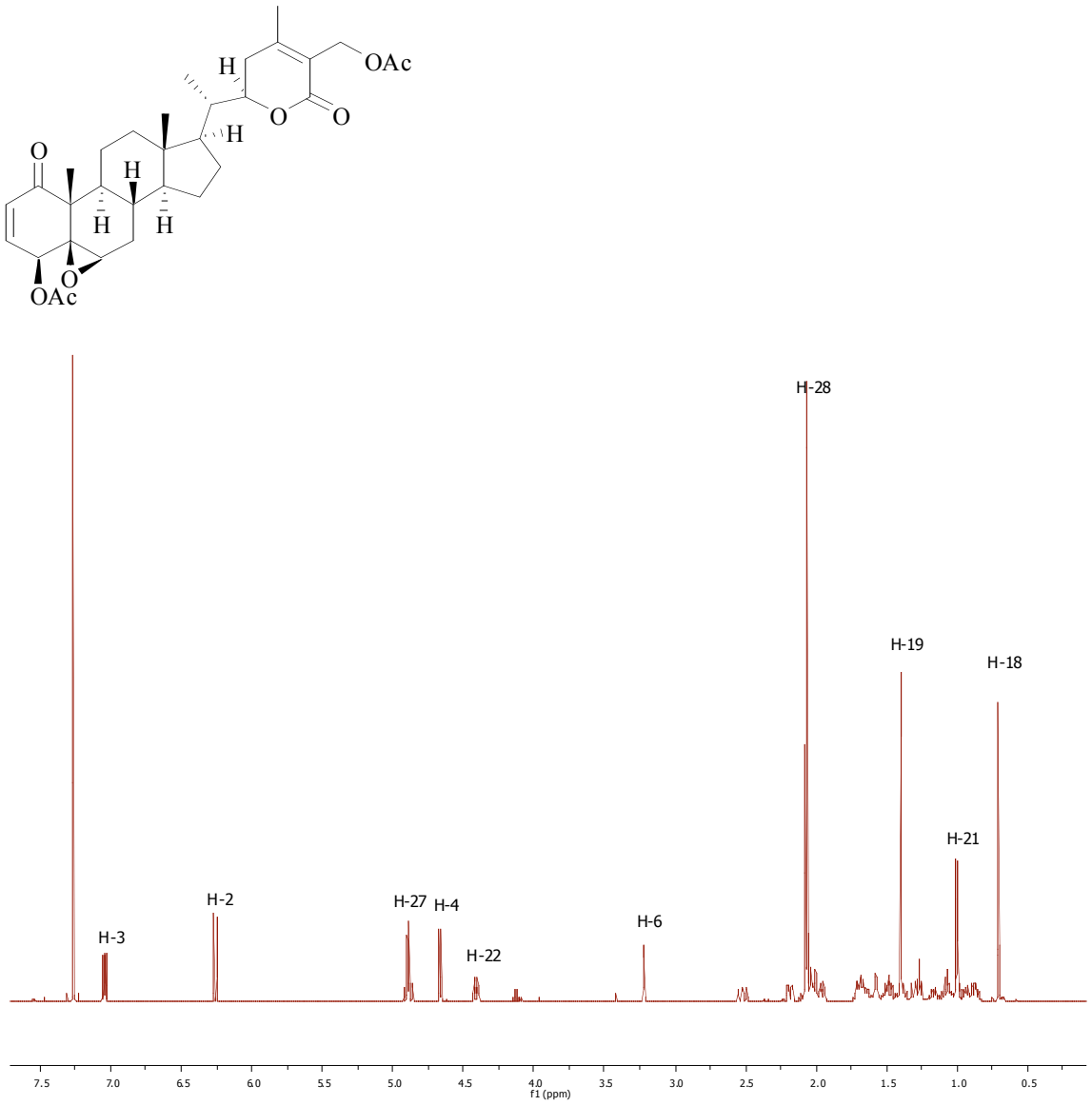


Figure 2.13. ORTEP view of 2,3-dihydro-27-deoxywithaferin A (**1$_b$**) from X-ray diffraction data.

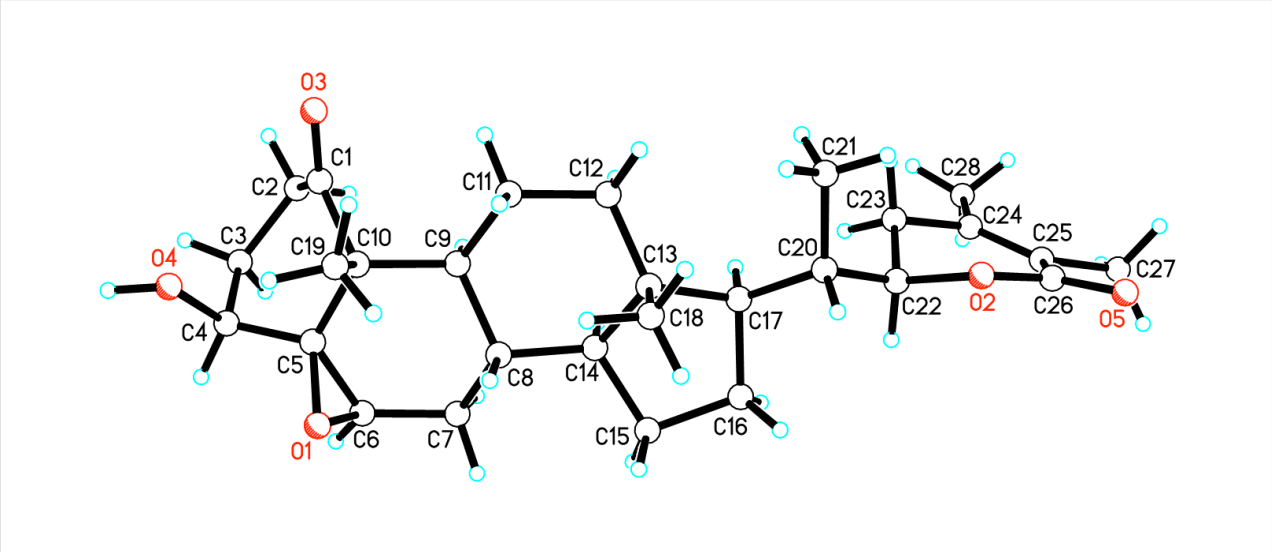


Table 2.3. ^{13}C NMR data[a] for 2,3-dihydrowithaferin A (**1_a**), 2,3-dihydro-27-deoxywithaferin A (**1_b**) and diacetylwithaferin A (**1_c**).

Positions	**1_a**	**1_b**	**1_c**
1	210.3	210.3	201.0
2	31.8	31.8	133.6
3	26.5	26.2	139.5
4	72.2	72.2	72.0
5	65.8	65.7	61.2
6	58.3	58.3	60.1
7	29.2	29.2	30.0
8	31.0	31.0	30.9
9	42.5	42.5	44.0
10	50.0	50.0	48.1
11	21.1	21.2	21.3
12	27.3	27.2	27.1
13	42.3	42.3	42.7
14	55.9	55.6	56.1
15	23.8	23.8	24.0
16	38.7	38.6	39.1
17	51.4	51.3	51.7
18	11.3	11.2	11.4
19	15.0	15.1	15.1
20	38.1	38.1	38.1
21	13.3	13.3	13.3
22	78.2	78.0	77.9
23	28.9	28.8	28.9
24	152.0	150.1	157.2
25	125.2	122.0	121.9
26	167.0	168.6	165.2
27	57.5	12.6	57.9
28	20.0	20.5	20.5
C_4-C=O	-	-	170.0
C_4-CO-CH_3	-	-	20.8
C_{27}-C=O	-	-	170.4
C_{27}-CO-CH_3	-	-	20.8

[a] ^{13}C NMR data were recorded in $CDCl_3$ at 125 MHz.

2.9 Experimental

General Experimental Procedures

NMR spectra were recorded on either a Bruker DRX-400 with a qnp probe or on a Bruder AV-500 with a cryoprobe. ^{1}H and ^{13}C spectra of compound **1** were recorded using the residual protonated signal in the $CDCl_3$ solvent (δ_H 7.24) or the central peak of the $CDCl_3$ triplet (δ_C 77.00) as the internal standard. Chemical shifts of compound **2** were expressed in ppm (d) using the residual protonated signal in the CD_3OD solvent at δ 3.31 (^{1}H) and δ 49 (^{13}C) as reference. Normal phase TLC was performed on Sorbent Technologies Silica G TLC plates (200 μm, w/UV 254) using the solvent system DCM-EtOAc-MeOH (1:8:1), and reverse phase TLC was performed on Sorbent Technologies C18 TLC plates (150 μm, w/UV 254) using H_2O-MeOH (1:1). Spots were visualized using UV light (254 nm) and spraying with vanillin-sulfuric acid reagent.

Bioassay Materials

Culture media, fetal bovine serum (FBS), penicillin G, streptomycin, MEM-nonessential amino acids, ribonuclease A, and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO). MEM-vitamin solution was purchased from Life Technologies, Inc. (Grand Island, NY). MTS reagents were purchased from Promega Co (Madison, WI). Annexin V was from BD Bioscience (Bedford, MA). Primary antibodies against total ERK and β-actin and secondary antibodies against mouse and rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies against phospho-Akt, total-Akt, phospho-ERK, caspase 3 and PARP were obtained from Cell Signaling Technologies (Beverly, MA). BCA protein assay

reagents were purchased from Pierce (Rockford, IL). Protease inhibitor mixture set II was purchased from Calbiochem (San Diego, CA).

Cell Culture and Media

The invasive oral squamous carcinoma cell lines, JMAR and MDA1986, were a gift to Dr. Mark Cohen from Dr. Jeffrey Myers (University of Texas, M.D. Anderson Cancer Center; Houston, TX). The head and neck squamous carcinoma cell line, UM-SCC-2, was a gift from Dr. Scott Weed (University of West Virginia, Morgantown, WV). The cells were grown in DMEM supplemented with 10% FBS, sodium pyruvate, nonessential amino acids, L-glutamine, a two-fold MEM-vitamin solution, and 1% penicillin/streptomycin. The laryngeal squamous carcinoma cell line, JHU-011, was kindly donated by Dr. Joseph Califano (Johns Hopkins University, Baltimore, MD). This was cultured in RPMI-1640, supplemented with 10% FBS and 1% penicillin/streptomycin. Adherent monolayer cultures were maintained on plastic plates and incubated at 37 °C with 5% CO_2.

Cytotoxicity Assay

The experiment was performed by a MTS assay according to the manufacturer's instructions. In brief, **1** was dissolved in dimethylsulfoxide (DMSO) and then diluted with culture media to prepare serial concentrations while the controls received only DMSO (<0.1%, v/v). The cells were seeded in 96-well microtiter plates (2×10^3 cells/well) in 100 μL of growth medium. After incubation for 24 h, varying levels of plant extracts or **1** were added to each well, and incubated

for 72h. Then, 20 μL MTS solution were added. After 3 h of incubation at 37 ^{o}C (5% CO_2), absorbance was measured at 490 nm with a microplate reader. Percentage viability was calculated as a fraction of the negative control (culture medium only), with data plotted as a function of % cell viability vs. concentration of **1**. The half-maximal inhibitory concentration (IC_{50}) was obtained from the MTS viability curves using GraphPad Prisim 5. All studies were performed in triplicate. In parallel with wells of the test samples, negative control wells with predetermined cell numbers were used to normalize the absorbance readings and to provide internal confirmation that the assay was linear over the range of absorbance and cell numbers measured.

Western Blot Analysis

After treatment cells were lysed (0.5% Nonidet P-40, 100 mM, 10 mM Tris (pH 7.5), 1:500 protease inhibitor mixture set II, 1 mM NaF, and 1 mM sodium orthovanadate) they were placed on ice for 20 min. Clear lysates were obtained by centrifugation (14,000 $\times g$ for 20 min). Equal amounts of protein were separated by SDS-PAGE and electro-transferred onto a Hybond nitrocellulose membrane (Amersham). The membranes were blocked and probed with the appropriate dilution of primary antibody overnight at 4 ^{o}C. The blots were washed three times in PBS-Tween-20 for 10 min, and then incubated in horseradish peroxidase-conjugated secondary antibody in PBS-Tween-20 at room temperature for 1 h. After washing in PBS-Tween-20, the proteins were visualized by enhanced chemiluminescence reagent (Amersham) and captured on Kodak XAR-5 film (Eastman Kodak, Rochester, NY). Where indicated, the blots were re-probed with antibody against β-actin to ensure equal loading and transfer of proteins.

Cell Cycle Arrest Study

After treatment, cells were trypsinized and 1×10^6 cells washed with 0.9% NaCl and fixed with 70% cold ethanol for 30 min at room temperature. After centrifugation (700 ×*g*; 5 minutes) cells were stained with propidium iodide (50 μg/mL in PBS) for 30 min. Then, cells were treated with DNAse free RNAse (1 mg/mL) for 30 min and analyzed by a Becton Dickinson LSRII Flow Cytometer.

Apoptosis Analysis

In order to evaluate the induction of apoptosis cells were co-stained with annexin V-propidium iodide (PI) and analyzed with flow cytometry. An analysis of phosphatidylserine on the outer leaflet of apoptotic cell membranes was performed using annexin V-FITC and PI to distinguish between apoptotic and necrotic cells. After treatment, cells (1×10^6 cells/ml) stained with annexin-V and PI according to manufacturer's instruction (BD Pharmingen, San Diego, CA). To each tube, 400 μL of 1x annexin binding buffer was added and stained cells were analyzed using a flow cytometer (BD LSRII; Becton Dickerson, San Diego, CA).

Statistical Methods

Statistical analysis was carried out using student t-test and Fisher's exact test. Means were calculated from three independent experiments.

III. WITHANOLIDES ISOLATED FROM *WITHANIA SOMNIFERA*

3.1 Introduction

In order to establish withaferin A anticancer structure activity relationships, additional amounts of withaferin A and a series of its analogues were highly desired. As the original source of plant material *Vassobia breviflora* from Argentina was no longer available for isolation of withaferin A, we searched for an alternative source of this compound. After conducting a thorough literature search, we chose *Withania somnifera* Dunal (Solanaceae) as our commercial source of the desired material. Because withaferin A was one of the major compounds in *W. somnifera*, and more than 70 other withanolides from this plant have been reported in the literature,[25] This species was the best target for our purpose. Recently, more unusual withanolides were isolated and identified from aeroponically grown *W. somnifera*, including compounds with sulfate,[26] uracil and adeninyl[27].

Withania somnifera, commonly known as "Ashwagandha" and widely cultivated in the drier parts of India, is well known and has a long history of use in Ayurvedic medicine. This "Indian Ginseng" has medical use nearly in all disorders that affect human health. In addition, it is used as general body tonic and expected to promote mental health, rejuvenate the body in debilitated condition and increase longevity.[28] Withaferin A and other withanolides from this plant are also reported to be responsible for its anticancer activities. [29]

Withanolides are a group of naturally occurring C_{28}-steroidal lactones built on an intact or rearranged ergostane scaffold in which C-22 and C-26 are appropriately oxidized for forming a δ-lactone ring on the nine-carbon side chain. These compounds are mainly found in members of the plant family Solanaceae and named after *Withania somnifera* from which the first withanolide, withaferin A, was isolated. Beside anticancer, withanolides are known to possess various other biological benefits including anticarcinogenic,[30] antioxidant,[31] adaptogenic,[32] antiparkinsonism,[33] antibacterial,[34] anti-inflammatory,[35] immunomodulatory[36] and antidepressant[37] effects.

From this plant, we isolated withaferin A and 10 related withanolides also shown in Figure 3.1. compounds isolated included 6α-chloro-5β,17α-dihydroxywithaferin A (**3**), (22*R*)-5β-formyl-6β,27-dihydroxy-1-oxo-4-norwith-24-enolide (**4**), 2,3-dihydrowithaferin A (**5**), 3-methoxy-2,3-dihydrowithaferin A (**6**), 2,3-didehydrosomnifericin (**7**), withanone (**8**) and 6α-chloro-5β-hydroxywithaferin A (**9**), withanolide A (**10**), withanoside IV (**11**) and withanoside X (**12**). Compound **3** was determined as a new structure and compound **4** was isolated from *W. somnifera* for the first time.[38] Several analogs of withaferin A were also prepared in this study for SAR and pre-clinical evaluations. All withanolides were initially screened for anti-proliferative activity using the MTS assay.

3.2 Botany and Collection of *W. somnifera*

W. somnifera raw plant material and extracts are currently sold as dietary supplements in the USA. To isolate large amounts of desired compounds, starting with plant extracts saved time, money, and effort on the plant extraction process. According to Jayaprakasam et al. (2003), this research group isolated considerable quantities of withaferin A and other withanolides from the plant extract of *W. somnifera* provided by PhytoMyco Research Corporation, Greenville, NC. We, therefore, purchased 200 g of plant extract from the same company in March 2009. According to the source, the extract was prepared by sequential extraction of the dried and ground leaves of *W. somnifera* with a mixture of CH_2Cl_2-MeOH (1:1) followed by MeOH and H_2O to obtain three fractions. All fractions were then combined, filtered and dried under reduced pressure. The purchased original extract has been stored at -20 °C in our laboratory to serve as a reference material. Authentication documentation that accompanied the sample has been deposited in the natural product chemistry lab at The University of Kansas.

3.3 Isolation of Withanolides from *W*. somnifera.

The powdered, dried plant extract (189 g) of *W. somnifera* was dissolved in 2 L of water to form a suspension. The water layer was placed in a large separatory funnel to which 2 L of hexane were added in order to remove the non-polar lipophilic organics such as fatty acids. The hexane layer was subjected to two additional liquid-liquid partitions. The hexane layers were combined and concentrated under reduced pressure to obtain a total of 32.6 g of a crude hexane extract. The water layers were each further placed in the separatory funnel to which 2 L of EtOAc were added in order to extract organic compounds of mid-polarity. The extraction process was

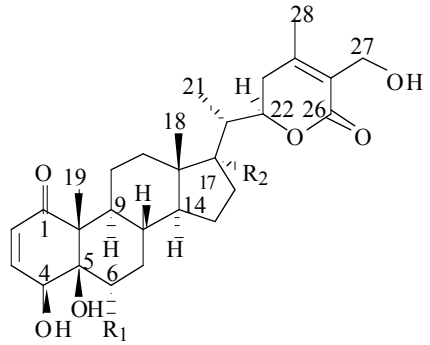
repeated twice and the organic layers were combined and concentrated under reduced pressure to yield a total of 32.9 g of a crude EtOAc extract. Subsequently, the water layers were each extracted with BuOH three times (2 L × 3) to afford a total of 80.0 g of a crude BuOH extract, which contained most of the polar organics. After all the described extraction procedures were completed, the aqueous layers were discarded.

The EtOAc extract was then subjected to passage over a Si gel column (600 g, 32~63 μm, Sorbent Technologies), eluted sequentially with acetone - CH_2Cl_2 solvent gradient (0-100%) followed by MeOH, to give eight major fractions (Fraction A-H; **Table 3.1**). Fraction F was further chromatographed on a Si gel (200 g, 12~26 μm, Sorbent Technologies) MPLC column, eluted with CH_2Cl_2-EtOAc-MeOH (40:10:1), to afford four additional fractions (F1-F4; **Table 3.2**). Fraction F2 was applied onto a C-18 reverse phase column (100 g, Sorbent Technologies), eluted with MeOH-H_2O (3:7) to remove the green color. The colorless fraction F2 was then separated on a Sephadex LH-20 column (200 g, GE Healthcare), eluted with MeOH to obtain the major components, and then purified by separation over a Si gel column (50 g, 12~26 μm, Sorbent Technologies), eluted with CH_2Cl_2-MeOH-EtOAc (18:1:1), to yield **3** (5 mg). HPLC chromatogram of **3** is shown in **Figure 3.2**. Fraction F1 was purified on a Sephadex LH-20 column (200 g, GE Healthcare), eluted with MeOH, to afford **7** (21mg). Compound **8** (800mg) was obtained as fine needles by recrystallization of fraction D from $CHCl_3$-acetone (1:1). Fraction E was chromatographed on a Si gel column, eluted with CH_2Cl_2-acetone (10:1), to collect twelve fractions (E1-El2, **Table 3.3**), and E6 was identified as **1** (34 mg). The major withanolide in fraction E7 was obtained as a white powder yielding **6** (22 mg) by recystallization from $CHCl_3$-acetone (1:1). E12 was separated over a C-18 reverse phase SPE column (5 g, 20 ml,

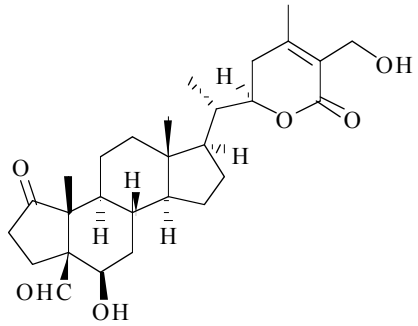
Phenomenex strata C-18), eluted with MeOH-H_2O (1:1), to give a mixture of two compounds (6.0 mg). Further purification of the mixture by HPLC using isocratic elution with acetonitrile-water (45:55) provided **4** (1.5 mg) and **5** (1.2mg). Compound **9** (7.3 mg) was purified from fraction E11 by a C-18 reverse phase SPE column (5 g, 20 mL, Phenoment strata C-18) using MeOH-H_2O (1:1). Compound **10** (521 mg) was purified as white powder by recrystallization of fraction C from $CHCl_3$-MeOH (1:1).

The BuOH extract was subjected to passage over a Si gel column (2 kg, 12~26 μm, Sorbent Technologies), eluted sequentially with MeOH-CH_2Cl_2 solvent gradient (30%- 100%), to give 21 fractions (**Table 3.4**). Fraction 4-5 was purified on a Sephadex LH-20 column (200 g, GE Healthcare), eluted with MeOH-H_2O (8:2), to afford a withanoside-rich fraction. This faction was further purified by PTLC eluting with CH_2Cl_2-MeOH-H_2O (40:10:1) and semi-prep HPLC eluting with CH_3CN-H_2O (25:75) to afford two withanosides **11** (5.6 mg) and **12** (6.3 mg).

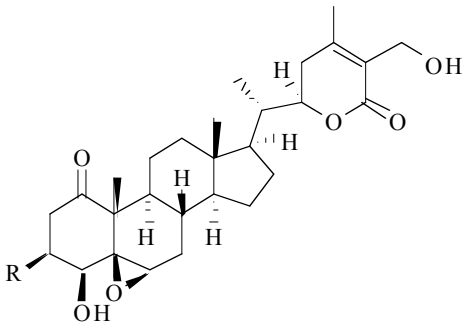
Figure 3.1. Withanolides and withanosides from *Withania somnifera*



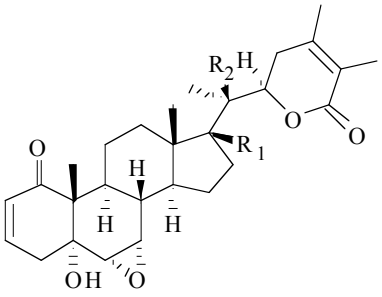
3 R_1=Cl; R_2=OH
7 R_1=OH; R_2=H
9 R_1=Cl; R_2=H



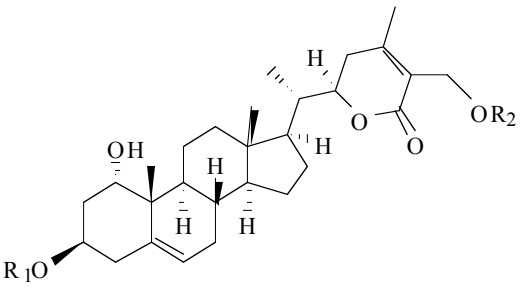
4



5 R=H
6 R=OMe



8 R_1=OH; R_2=H
10 R_1=H; R_2=OH



11 R_1=Glc-(1→6)-Glc; R_2=H
12 R=Glc; R_2=Glc

Table 3.1. Fractionation of EtOAc extract from *W. somnifera*.

Fractions	Wt. (in g)	%Yield[a]
A	4.2	12.8
B	3.8	11.5
C	6.7	20.4
D	3.3	10.0
E	6.0	18.2
F	1.2	3.6
G	1.4	4.2
H	3.2	9.7

[a]As a percentage of the EtOAc extract (32.9 g)

Table 3.2. Fractionation of Fraction F from *W. somnifera*.

Fractions	Wt. (in g)	%Yield[a]
F1	0.25	6.9
F2	0.31	8.6
F3	1.22	33.9
F4	1.12	31.1

[a]As a percentage of Fraction F (3.6 g)

Table 3.3. Fractionation of Fraction E from *W. somnifera*.

Fractions	Wt. (in g)	%Yield[a]
E1	0.21	3.5
E2	0.86	14.3
E3	0.73	12.2
E4	0.32	5.3
E5	0.22	3.7
E6	0.21	3.5
E7	0.48	8.0
E8	0.21	3.5
E9	0.45	7.5
E10	0.43	7.2
E11	0.13	2.2
E12	0.26	4.3

[a]As a percentage of Fraction E (6.0 g)

Table 3.4. Fractionation of BuOH extract from *W. somnifera*.

Fractions	Wt. (in g)	%Yield[a]
1	6.3	7.8
2	12.2	15.3
3	0.49	0.6
4	0.33	0.4
5	0.58	0.7
6	0.96	1.2
7	0.85	1.1
8	0.91	1.1
9	0.97	1.2
10	1.52	1.9
11	1.64	2.1
12	0.43	0.5
13	0.55	0.7
14	0.68	0.9
15	0.79	1.0
16	0.86	1.1
17	3.54	4.4
18	2.68	3.4
19	5.63	7.0
20	1.46	1.8
21	1.83	2.3

[a]As a percentage of the BuOH extract (80.0 g)

Figure 3.2. HPLC chromatogram of 6α-chloro-5β,17α-dihydroxywithaferin A (**3**).

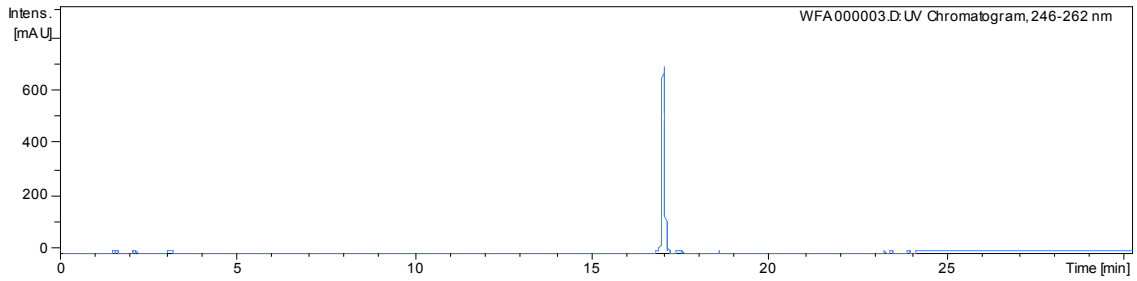
Mobile phase: A-water, B-Acetonitrile; isocratic 50% B for 30 min

Flow rate: 1 mL/minute

Injection Vol.: 10 µm

Injection Conc.: 1.0 mg/mL in Methanol

Detection: 254 nm



3.4 Structure Elucidation

The ^{13}C NMR data for compounds **3 - 12** are shown in **Table 3.5**, and data for proton NMR spectra have been presented in **Figures 3.3** through **3.14**. Fraction E6 was identified as withaferin A (compound **1** in Chapter II) by ^{1}H NMR and co-chromatography with compound **1** isolated from *Vassobia breviflora.*

Compound **3** was obtained as colorless parallelepiped-shaped crystals by recrystallization from MeOH-CH_2Cl_2-EtOAc (5:1:1), having the molecular formula $C_{28}H_{39}ClO_7$ as deduced from its HRESIMS (observed *m/z* 523.2440 $[M + H]^+$, calcd for $C_{28}H_{40}ClO_7$ 523.2463) and NMR data. The presence of chlorine was supported by the intensity of the $[M + H + 2]^+$ peak, which was approximately one-third as intense as the pseudomolecular ion peak $[M + H]^+$ (**Figure 3.4**). IR absorption bands at 3500, 1700, and 1650 cm^{-1} revealed the presence of OH, carbonyl and lactonic groups.

The ^{13}C NMR (DEPT) spectrum of **3** displayed 28 signals assigned to four methyl, seven methylene (including one oxygenated at δ 57.3, nine methane (including two olefins at δ 127.8 and δ 142.8), and eight quaternary carbons (including one keto carbonyl at δ 200.2 and one ester carbonyl at δ 167.0, two olefins at δ 154.2 and δ 125.2). The ^{13}C NMR (DEPT) spectrum of **3** displayed 28 signals assigned to four methyl, seven methylene (including one oxygenated at δ 57.3); nine methane (including two olefins at δ 127.8 and δ 142.8); and eight quaternary

carbons (including one keto carbonyl at δ 200.2 and one ester carbonyl at δ 167.0, two olefins at δ 154.2 and δ 125.2, two oxygenated at δ 84.8 and δ 78.1) and corresponding to $C_{28}H_{35}$. Thus, the remaining four hydrogen atoms were assigned to four OH groups. These data were consistent with the characteristic features of withanolides, C_{28}-steroidal lactones with a δ-lactone ring on the nine-carbon side chain.

The ^{1}H NMR spectrum of **3** (**Figure 3.3**A) also showed typical signals for a withanolide. After analyzing the COSY (**Figure 3.3**B), HSQC (**Figure 3.3**C), and HMBC (**Figure 3.3**D) spectra of **3,** the four methyl groups resonated at δ 0.99 (3H, d, J =7.0 Hz), δ 0.78 (3H, s), δ 1.27 (3H, s), and δ 2.03 (3H, s), corresponding to the secondary methyl of C-21 and the quaternary methyls at C-18, 19 and 28 of a withanolide.

Finally, the structure of **3** was confirmed through single-crystal X-ray diffraction experiments. The absolute configuration of **3** was confirmed on the basis of anomalous scattering of the chlorine atoms in the crystal (**Figure 3.5**). Thus, the structure of the new withanolide **3** was established as 6α-chloro-5β,17α-dihydroxywithaferin A. [38]

The known compounds were identified, by comparison of their NMR and MS data with those reported in the literature. The NMR data of compound **9** was very similar to those of compound **3.** The obvious difference between **3** and **9** was the presence of a quaternary carbon (δ 84.8) in **3** and a methine carbon (C-17) in the latter (^{1}H NMR δ 1.07, ^{13}C NMR δ 51.8), suggesting that **9**

(**Figure 3.11**) was a 17-dehydroxy derivative of **3**. This observation was supported by the high-frequency shift of C-13 (δ 48.3 in **3** and δ 43.2 in **9**), C-16 (δ 36.5 in **3** and δ 27.2 in **9**), and C-20 (δ 42.6 in **3** and δ 38.7 in **9**) in the ^{13}C NMR spectra. It was also supported by the ^{1}H-^{1}H COSY and HSQC spectra (a fragment CH_3-CH-CH(O)-CH_2- of the side chain linked to the oxygenated quaternary C-17 in **3** and a fragment CH_3-CH-CH(O)-CH_2- of the side chain linked to methine C-17 in **9**), and the HMBC correlations between H_3-21 (δ 0.99) and C-17 (δ 84.8), and between H_3-18 (δ 0.78) and C-17 (δ 84.8) in **3**. Finally, the structure of **9** was confirmed as 6α-chloro-5β-hydroxy withaferin A. The proton NMR of **7** (**Figure 3.9**) revealed that its structure is similar to **9**. The slight difference is that in **7**, the function group attached to C-6 was a hydroxyl group (C-6 in compound **7**, δ 75.2) instead of chlorine (C-6 in compound **9**, δ 66.7). Compound **7** was confirmed as 2,3-didehydrosomnifericin.

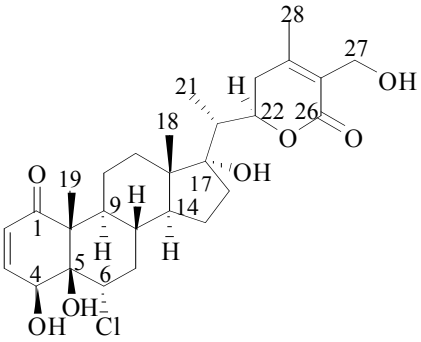
Compound **5** was established as a 2,3-dihydroxy derivative of withaferin A (**1**), which was the same as compound $\mathbf{1_a}$ obtained by the hydrogenation of withaferin A (see Chapter II). Its NMR spectrum has been presented in **Figure 3.7**. Compared to **5**, Compound **6** had an additional 3-methoxy group, which showed a strong signal at δ 3.35 (3H, s) as can be seen in **Figure 3.8**. The C-3 (δ 26.5) in the ^{13}C NMR spectrum of **5** shifted to δ 77.5, suggesting the oxygenated C-3 in compound **6**. Compound **6** was further confirmed as 3-methoxy-2,3-dihydrowithaferin A. Compound **4** (22*R*)-5β-formyl-6β,27-dihydroxyl-1-oxo-4-norwith-24-enolide was different from all the other withanolides isolated in that the A ring of **4** was an unusual five member ring. The H-4 (δ 9.53) in proton NMR of **4** (**Figure 3.6**) indicated the presence of an aldehyde in the structure.

In the low-field region of the proton NMR of compound **8** (**Figure 3.10**), withanone, there were two signals for vinylic hydrogens at δ 5.83 and δ 6.58, which corresponded to an α,β-unsaturated A ring. Two mutually coupled adjacent protons at δ 3.03 and δ 3.30 supported a 6α-7α-epoxy on the B ring. The proton NMR of compound **10** (**Figure 3.12**), which was later confirmed as withanolide A, was similar to that of compound **8**. However, the chemical shift of C-20 at δ 75.3 (δ 42.9 in **8**), C-17 at δ 54.6 (δ 84.6 in **8**) and the singlet of H-21 at δ 1.30 in compound **10** suggested the presence of a hydroxyl group attached to C-20 instead of C-17 in compound **8**.

Detailed analysis of the 1D and 2D NMR spectra and comparison with the reported data established that compounds **11** and **12** were withanosides containing two glucose units. The aglycones corresponding to **11** and **12** were determined as pubesenolide [(20*S*,22*R*)-1α,3β,27-trihydroxywitha-5,24-dienolide]. In compound **12**, the sugar moieties were two glucose monosaccharide residues as established by β-anomeric proton signals (**Figure 3.14**) at δ 5.08 (d, *J*=8.0Hz, H-1') and δ 5.09 (d, *J*=7.5Hz, H-1"). HMBC correlations of H-3 and C-1', H-1' and C-3, H-27 and C-1", as well as H-1" and C-27 suggested their linkage sites at C-3 and C-27 in compound **12**. However, in compound **11** (**Figure 3.13**), the HMBC experiment showed long-range correlations between H-1' and C-3, and H-1" and C-6'. Based on this information and reported data, **11** and **12** were confirmed to be withanoside IV and withanoside X.

Figure 3.3. 1D and 2D NMR spectra of 6α-chloro-5β,17α-dihydroxywithaferin A (**3**).

A. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of 6α-chloro-5β,17α-dihydroxywithaferin A (**3**).



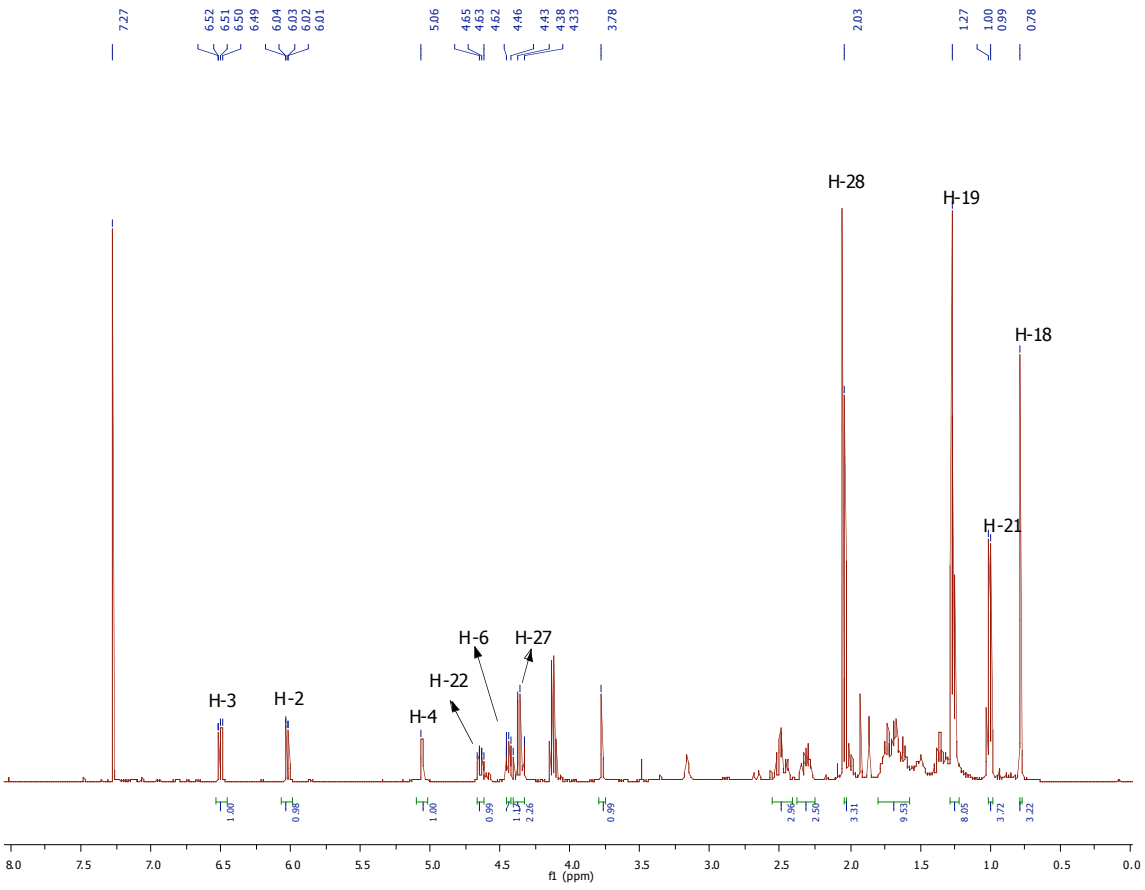


Figure 3.3. 1D and 2D NMR spectra of 6α-chloro-5β,17α-dihydroxywithaferin A (**3**) – **(**cont.)

B. COSY spectrum of compound **3**.

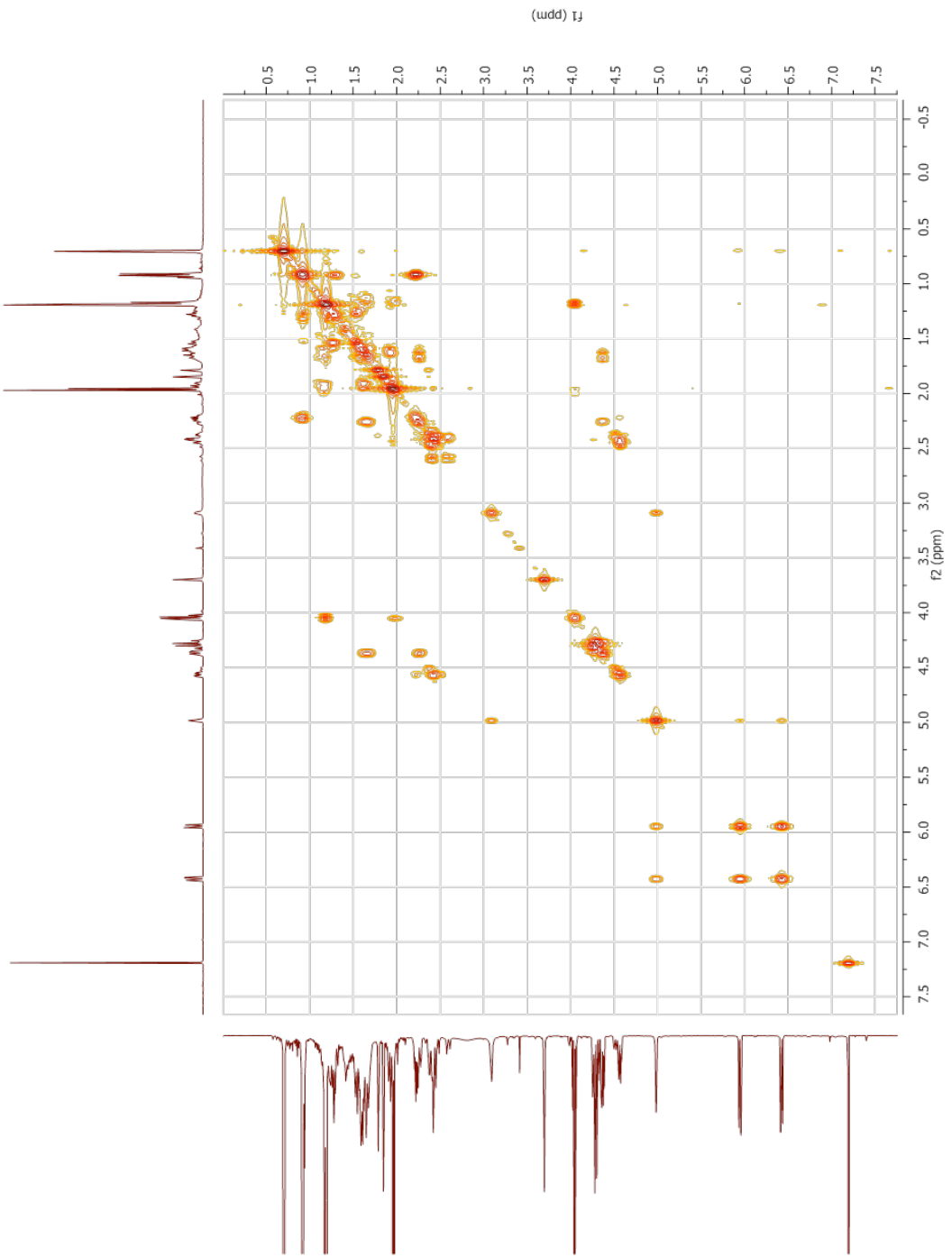


Figure 3.3. 1D and 2D NMR spectra of 6α-chloro-5β,17α-dihydroxywithaferin A (**3**) – **(**cont.)

C. HSQC spectrum of compound **3**.

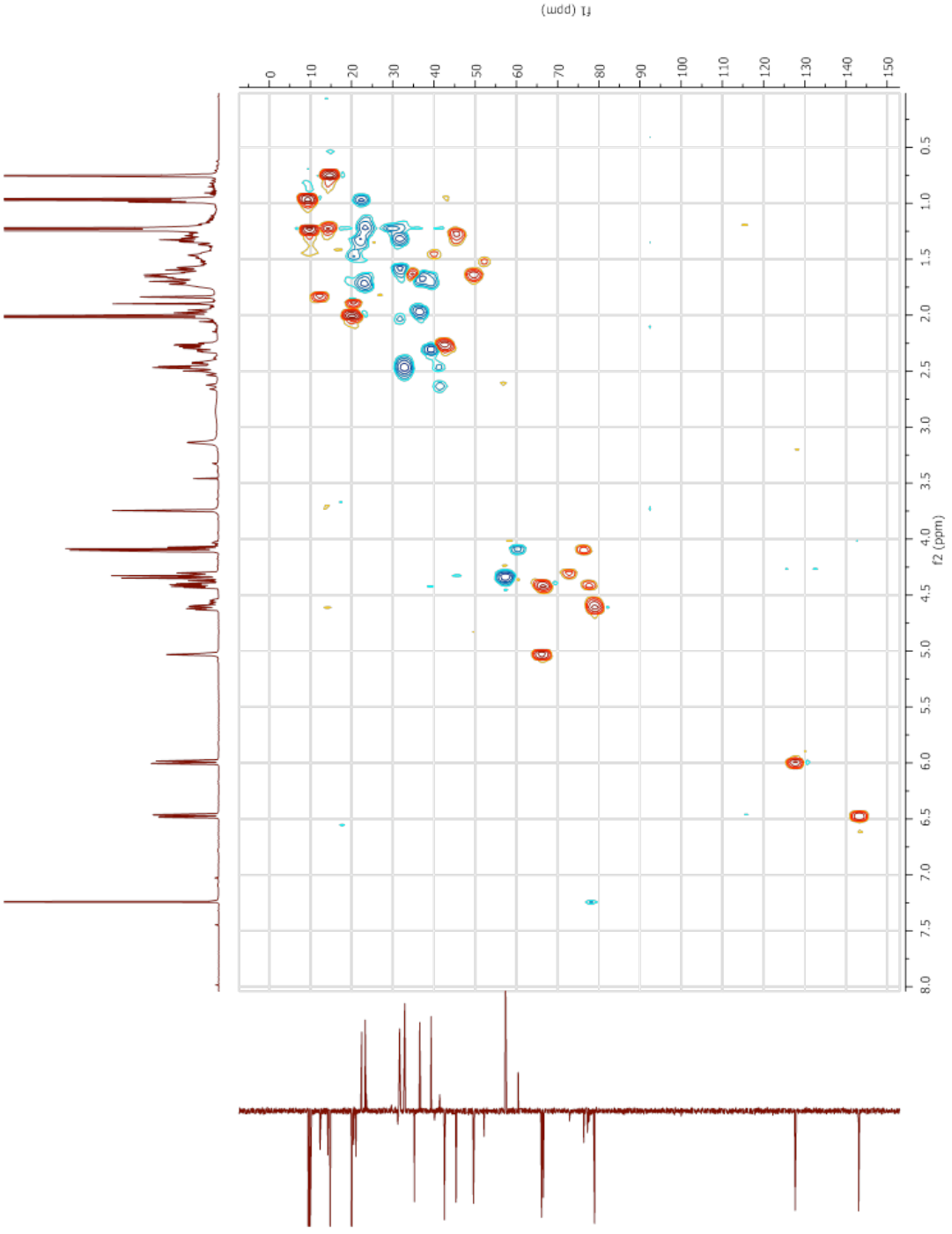


Figure 3.3. 1D and 2D NMR spectra of 6α-chloro-5β,17α-dihydroxywithaferin A (**3**) – **(**cont.)

D. HMBC spectrum of compound **3**.

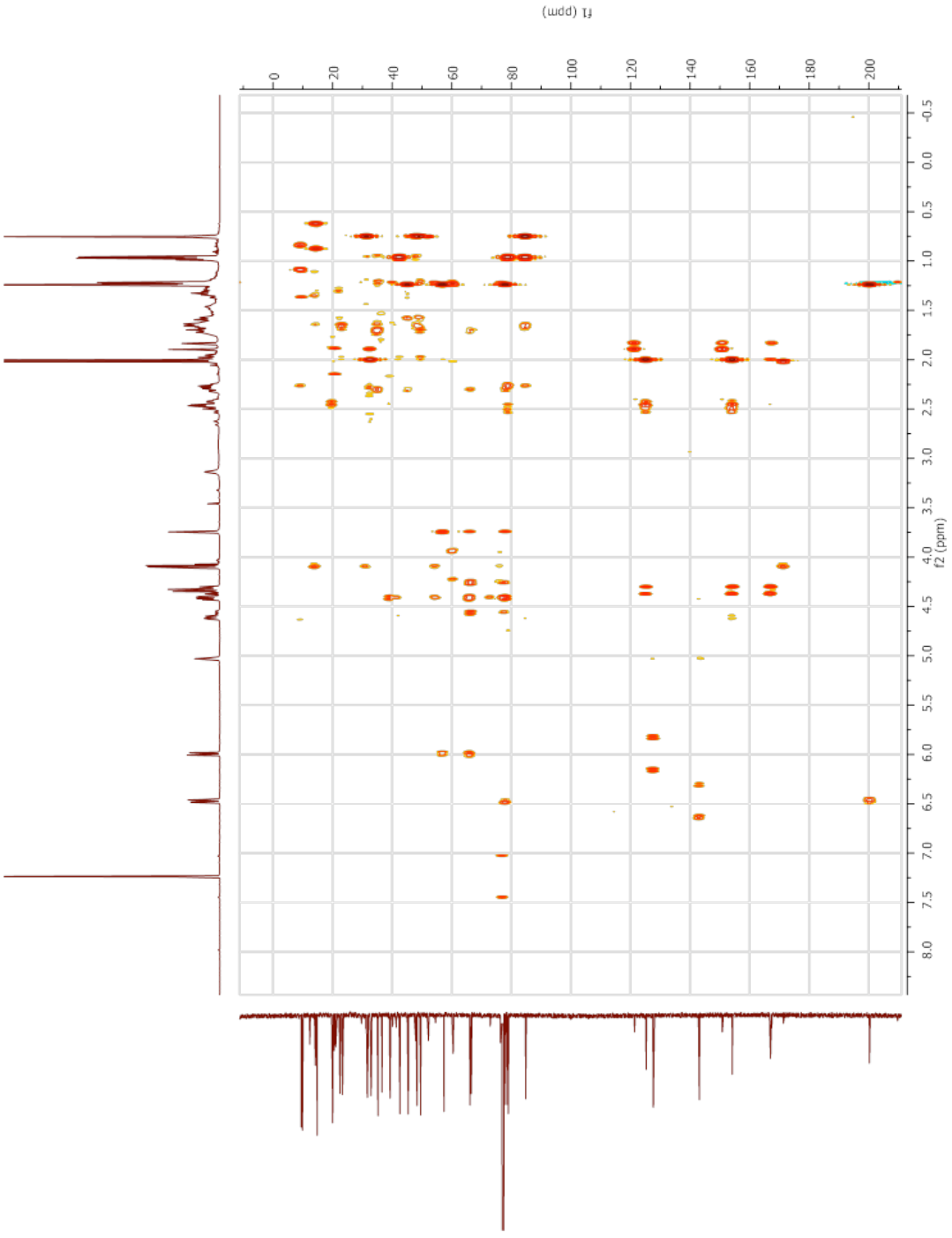
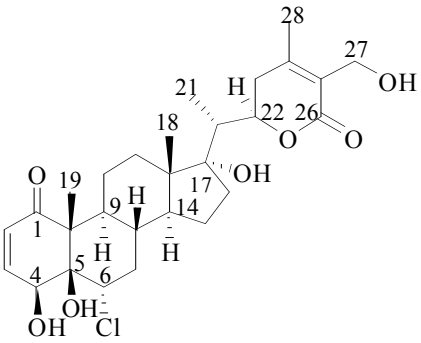


Figure 3.4. Mass spectrum of 6α-chloro-5β,17α-dihydroxywithaferin A (**3**).



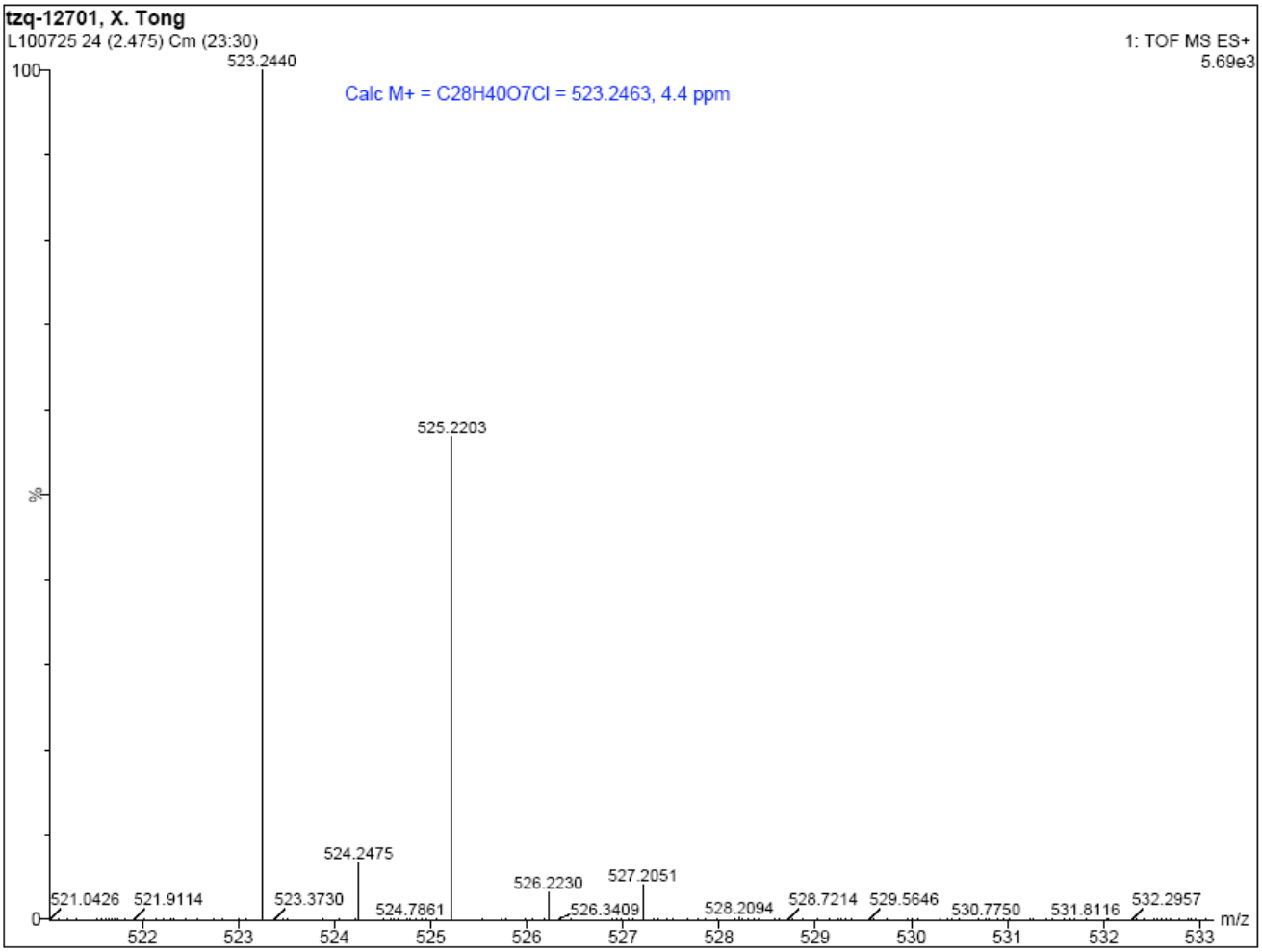


Figure 3.5. ORTEP view of 6α-chloro-5β,17α-dihydroxywithaferin A (**3**) from X-ray diffraction data.

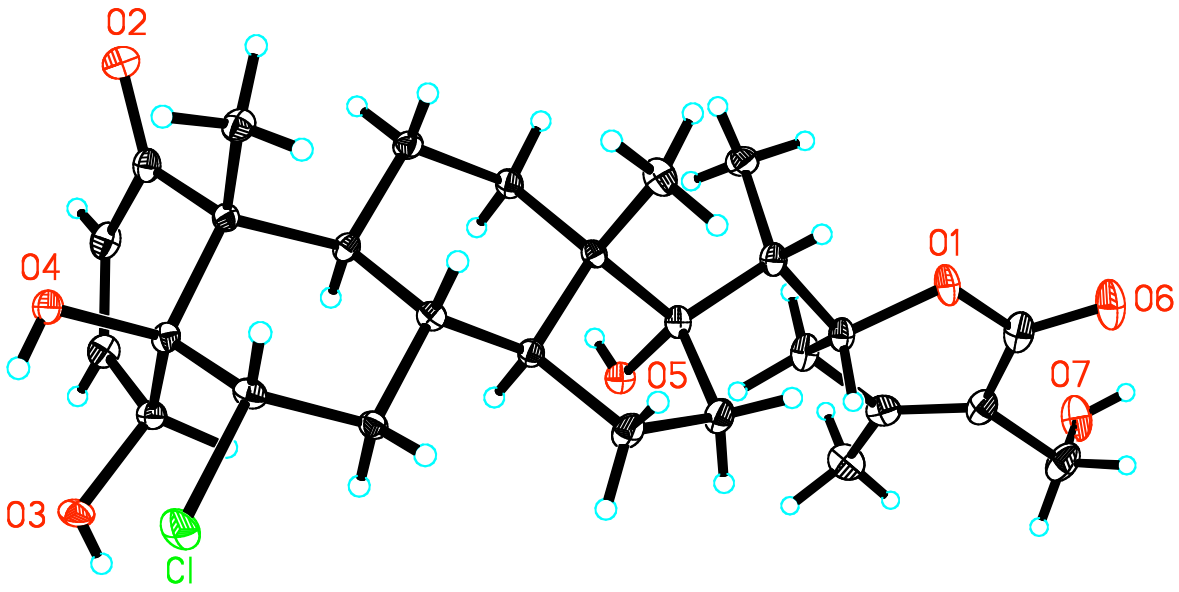


Figure 3.6. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of (22*R*)-5*β*-formyl-6*β*,27-dihydroxy-1-oxo-4-norwith-24-enolide (**4**).

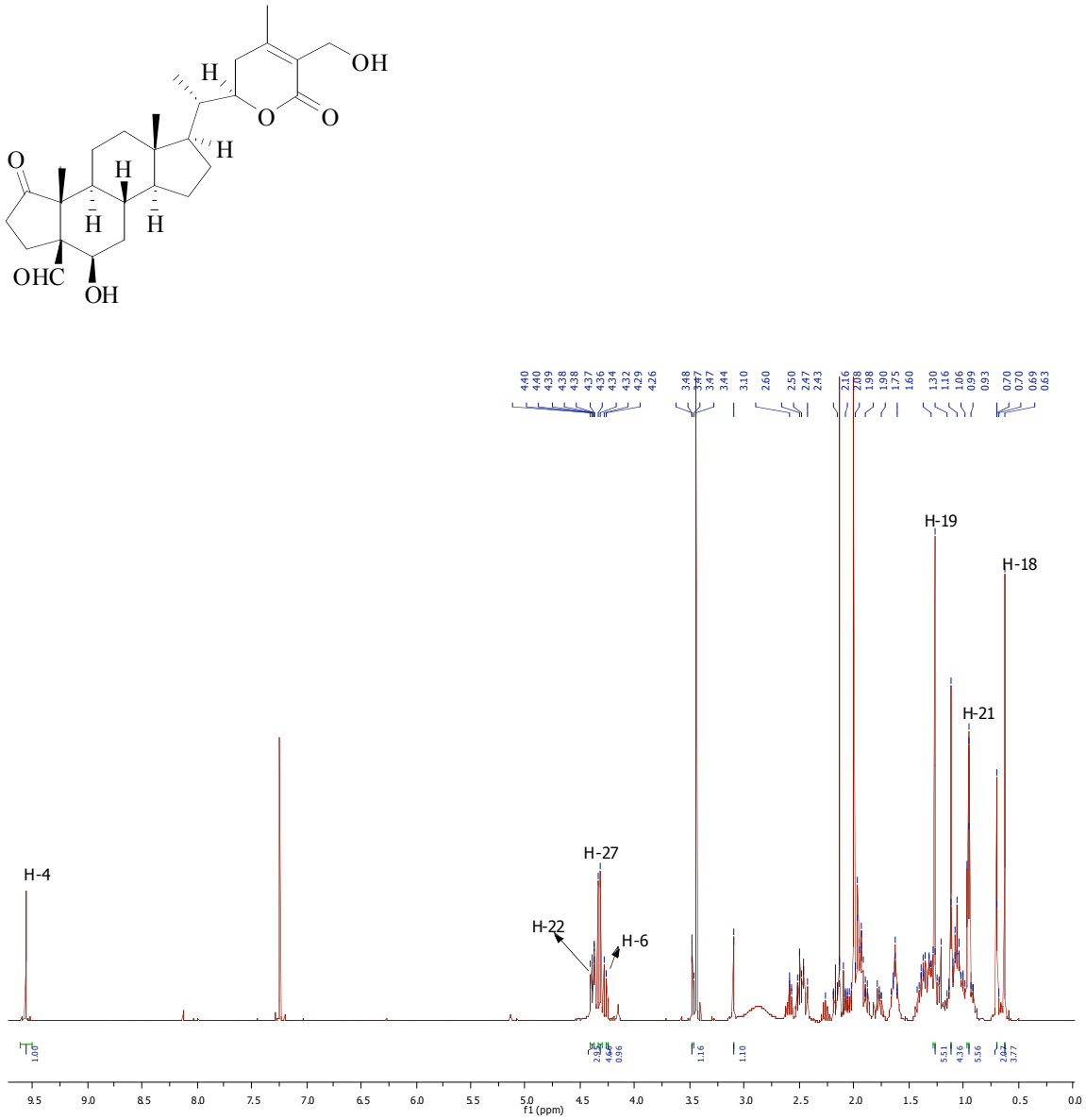


Figure 3.7. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of 2,3-dihydrowithaferin A (**5**).

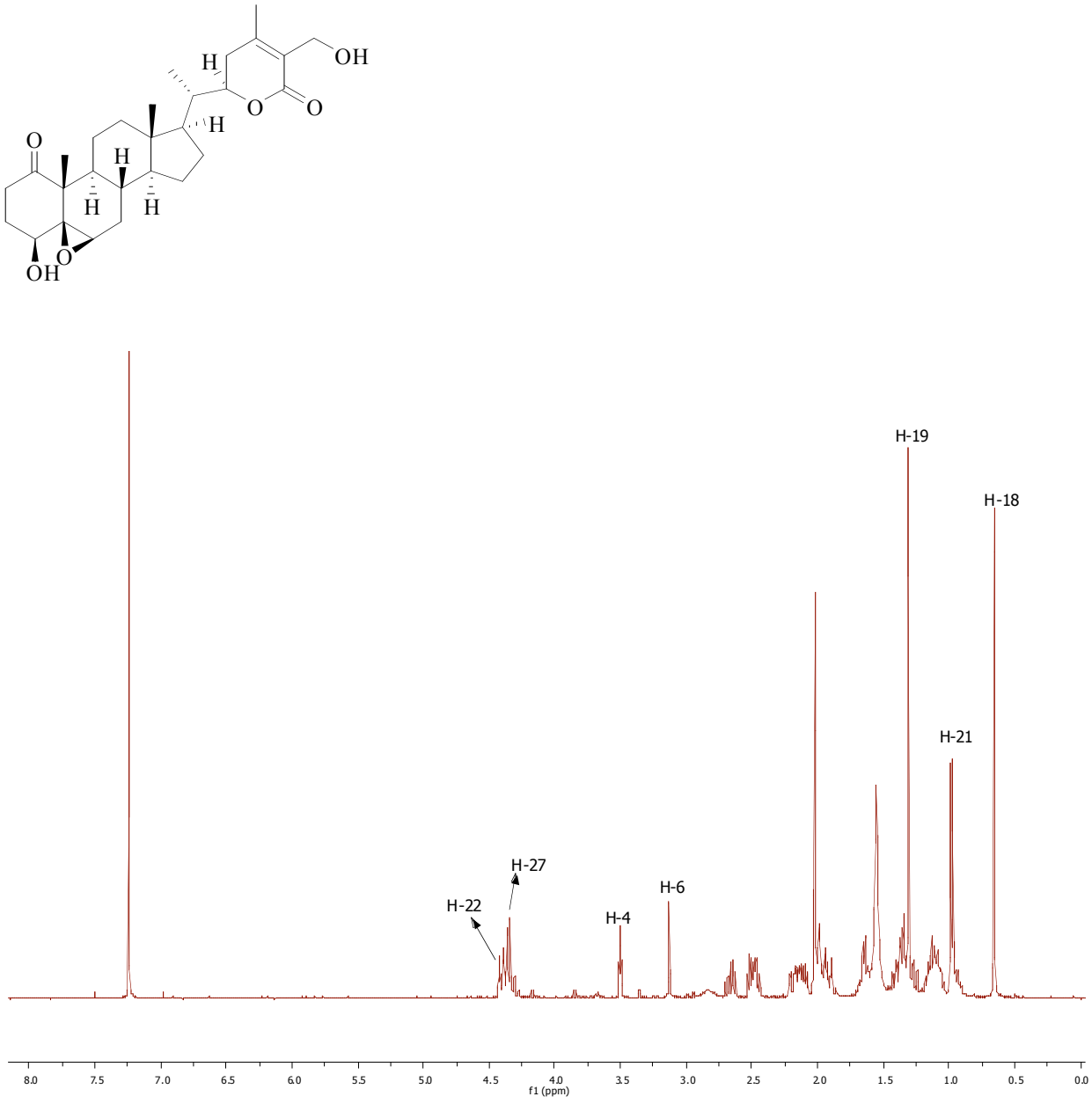


Figure 3.8. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of 3-methoxy-2,3-dihydrowithaferin A (**6**).

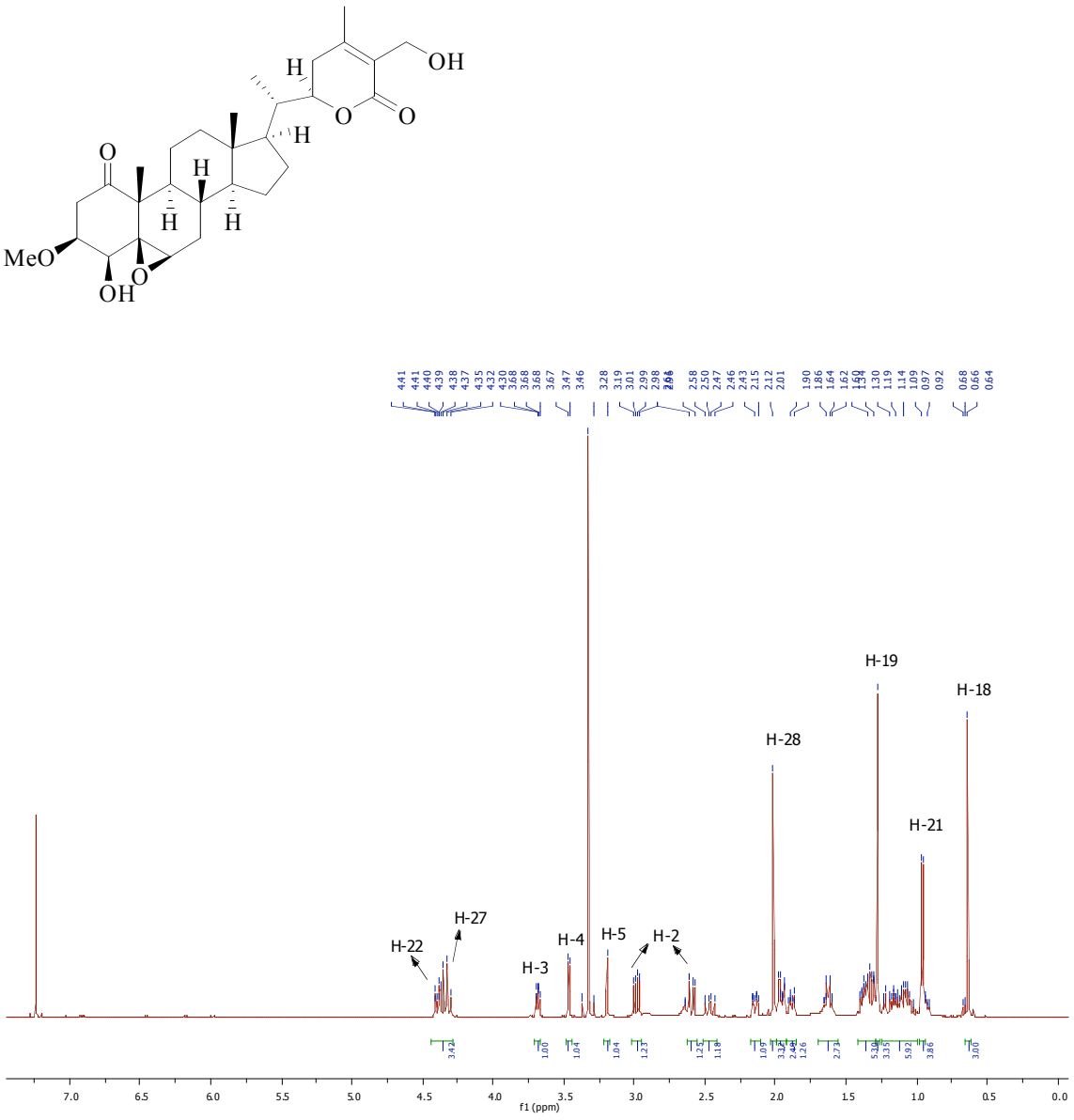


Figure 3.9. ^{1}H NMR spectrum (500 MHz, CD_3OD) of 2,3-didehydrosomnifericin (**7**).

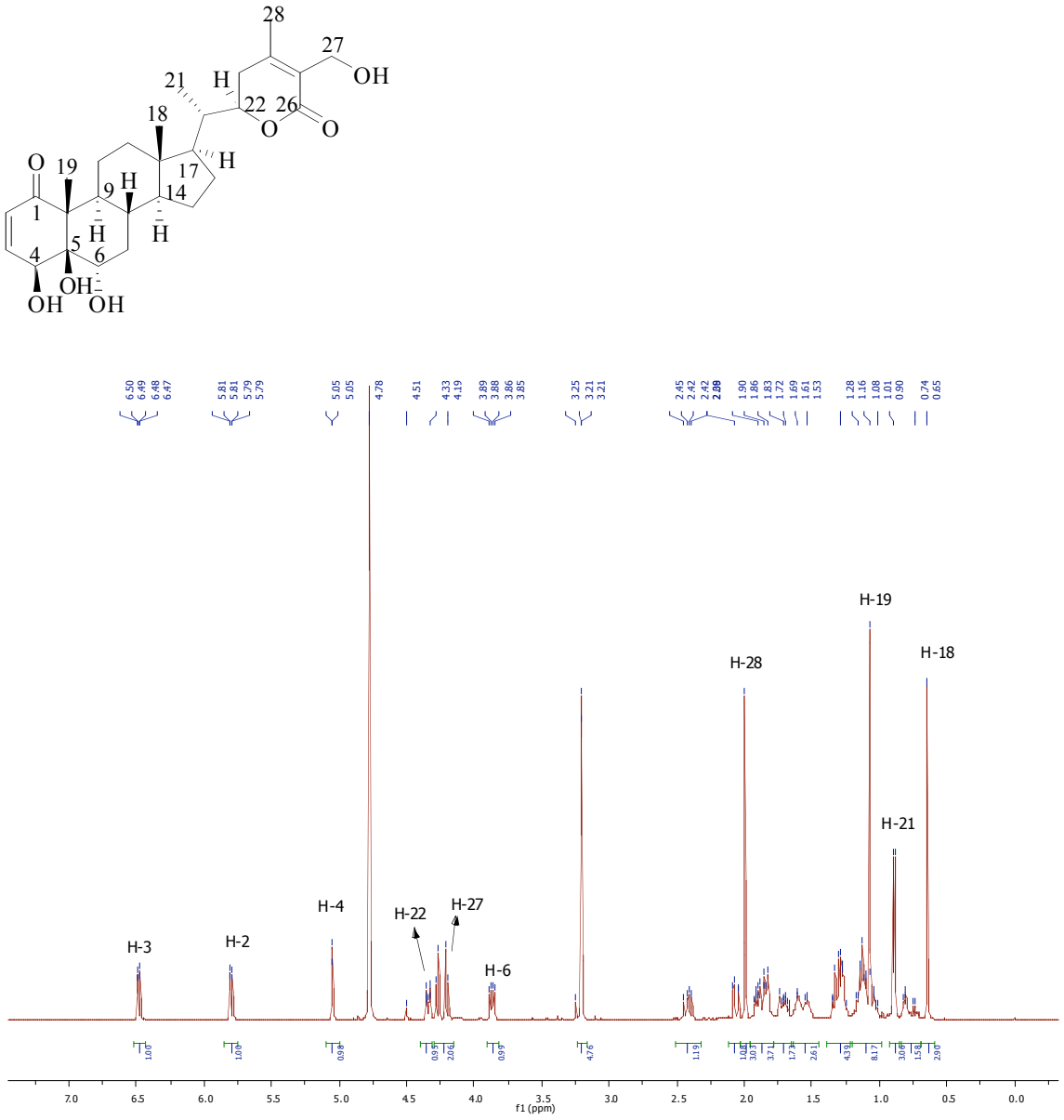


Figure 3.10. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of withanone (**8**).

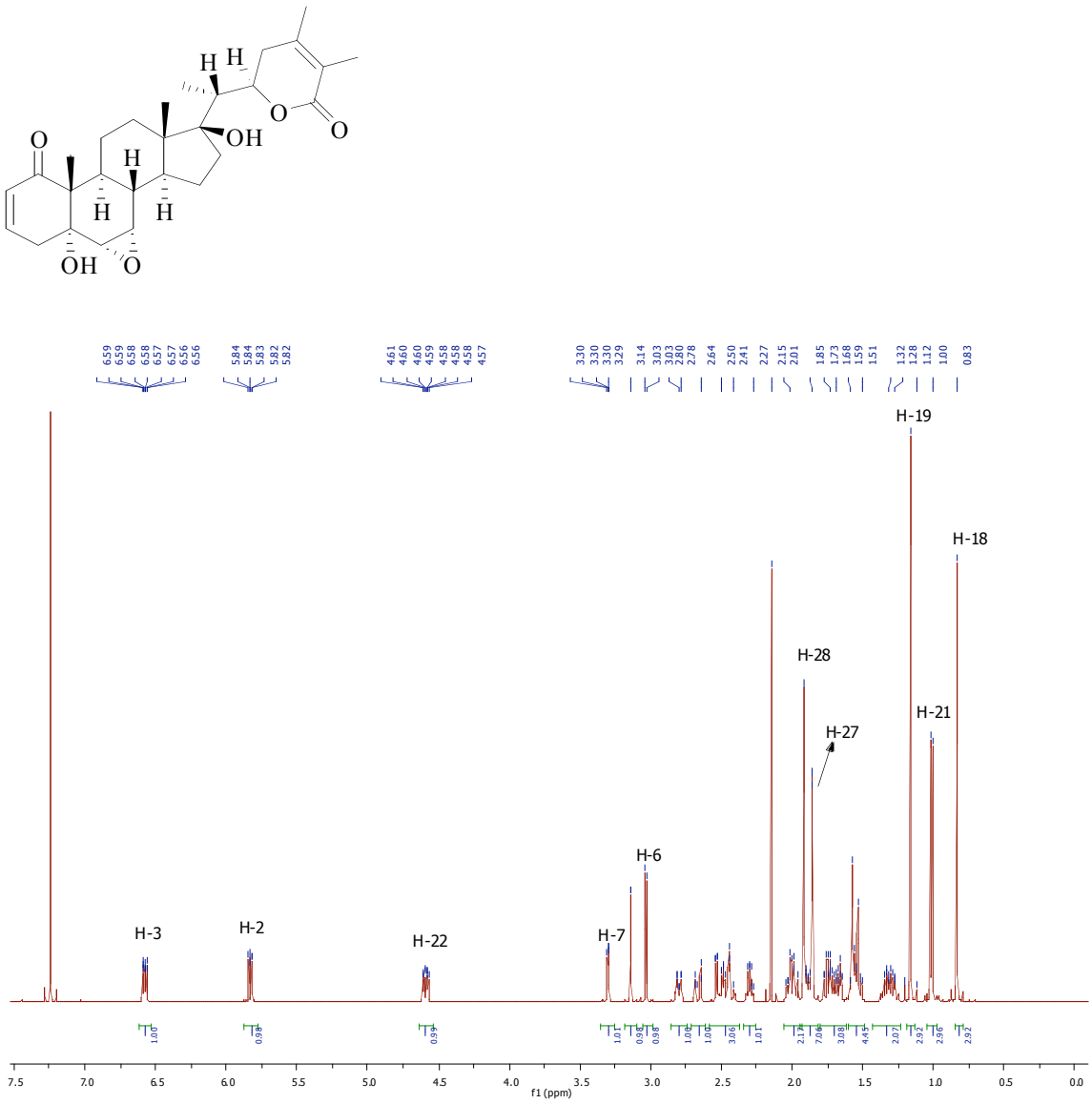


Figure 3.11. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of 6α-chloro-5β-hydroxywithaferin A (**9**).

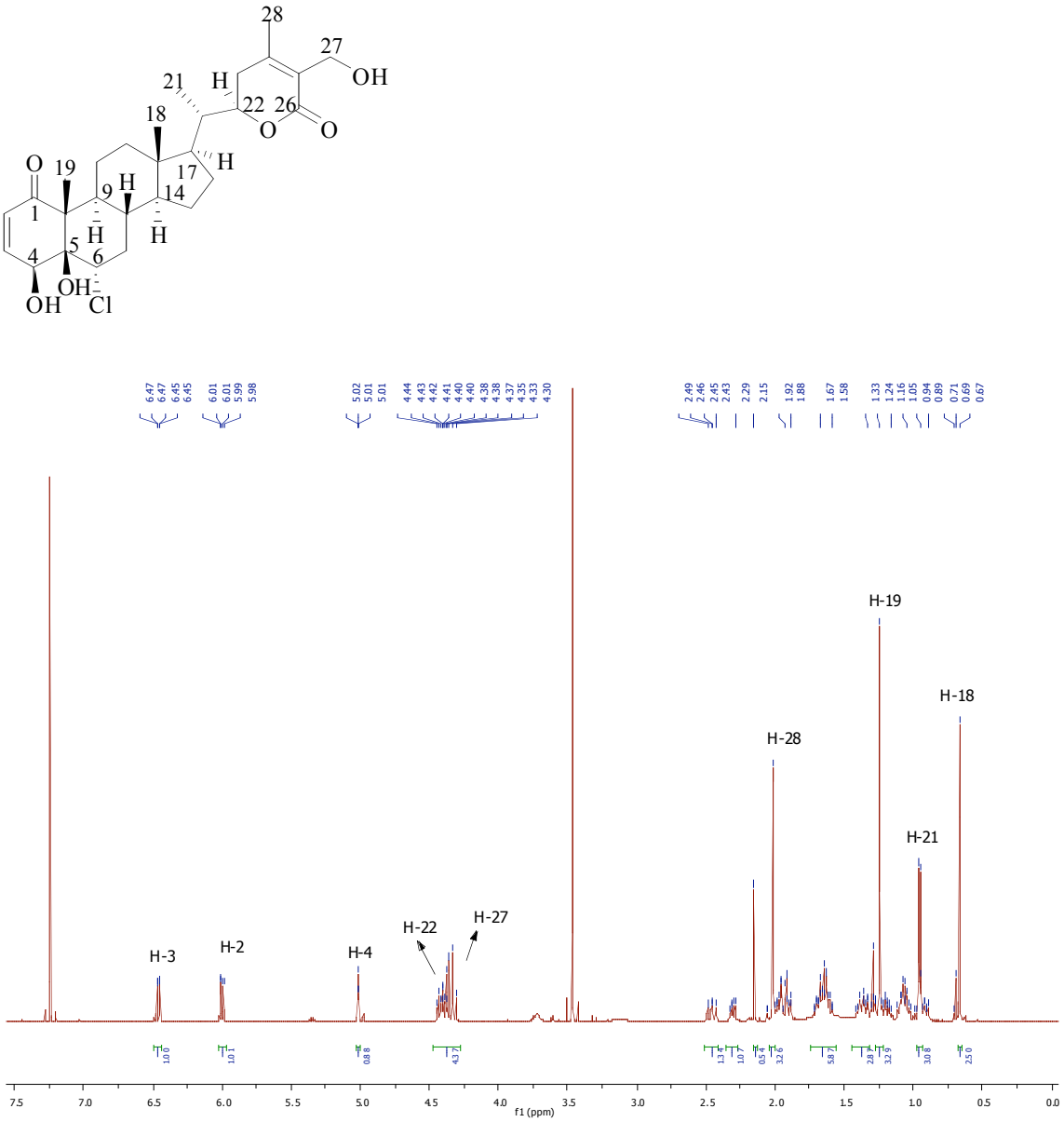


Figure 3.12. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of withanolide A (**10**).

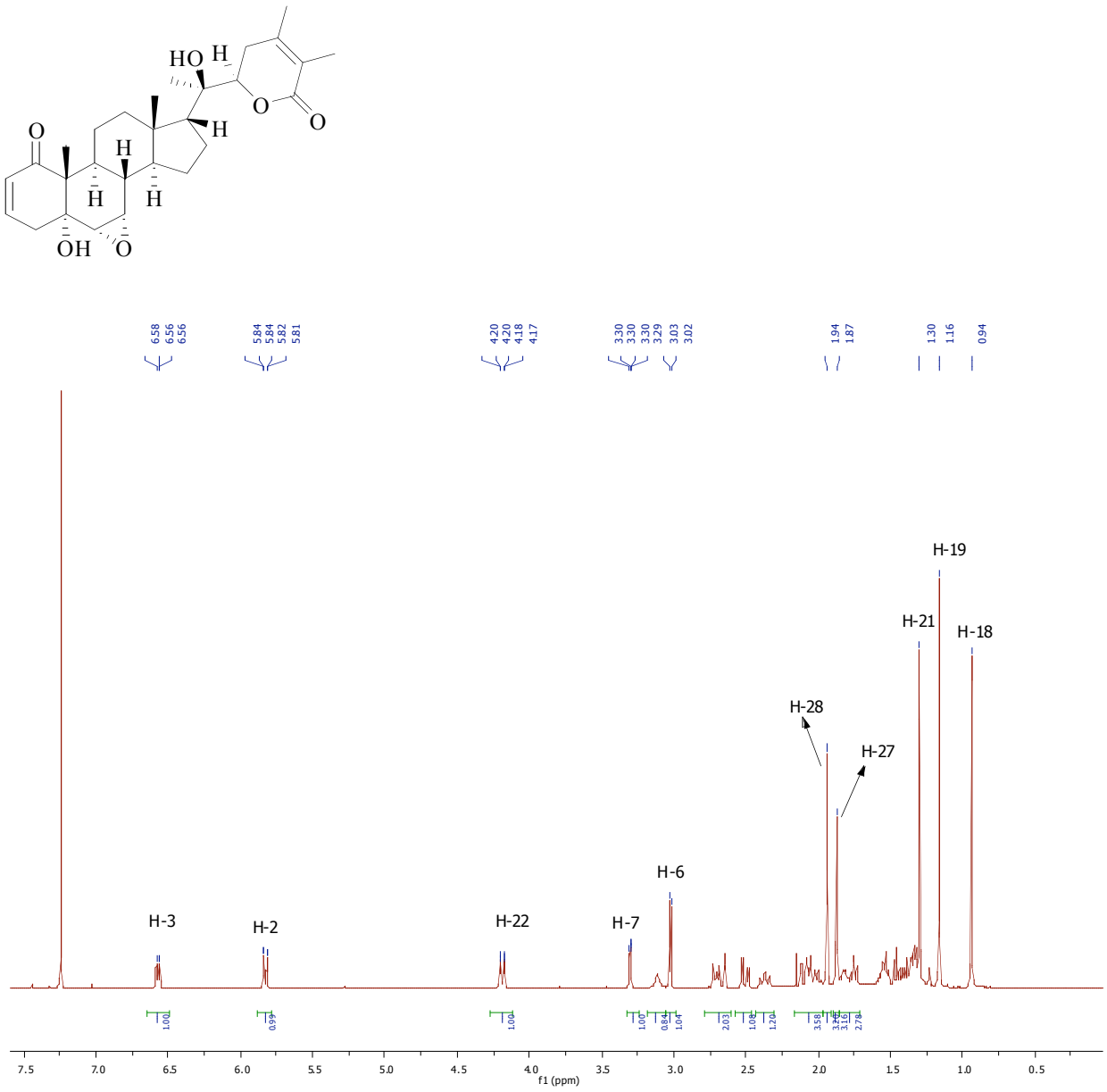


Figure 3.13. ^{1}H NMR spectrum (500 MHz, pyridine-d_5) of withanoside IV (**11**).

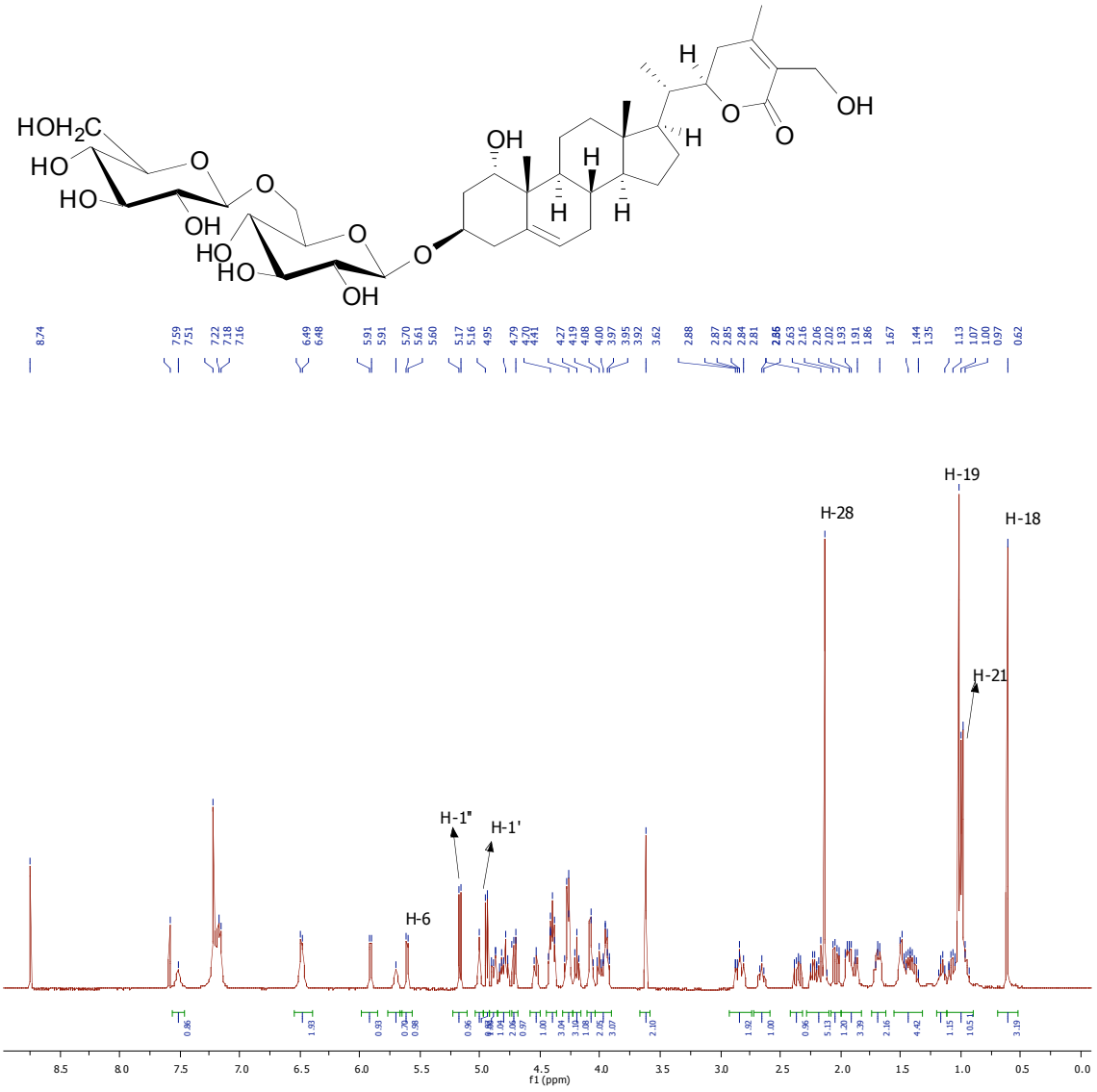


Figure 3.14. ^{1}H NMR spectrum (500 MHz, pyridine-d_5) of withanoside X (**12**)**.**

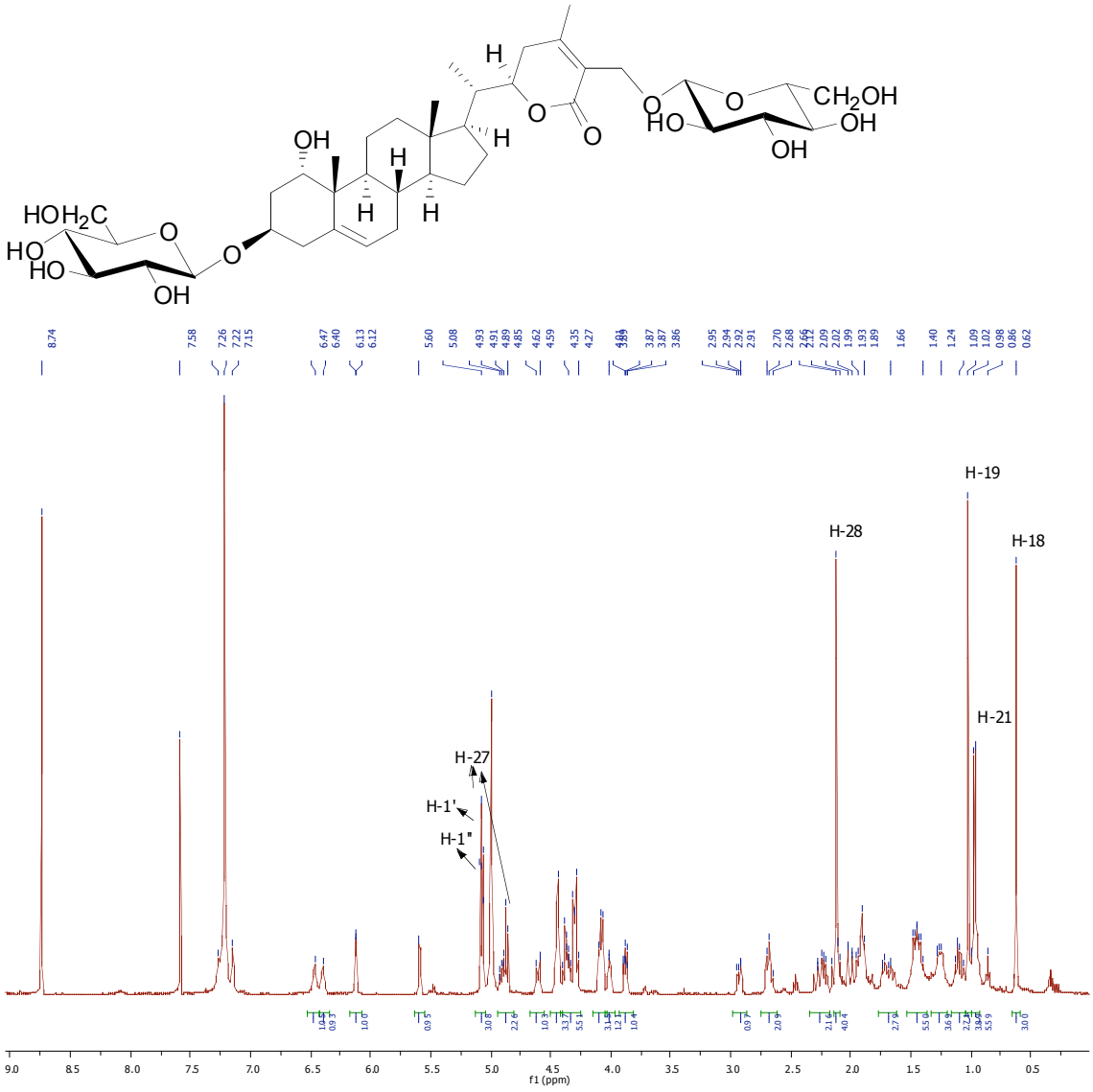


Table. 3.5. ^{13}C NMR data for compounds **3-12**.

	3a	4a	5a	6a	7b	8a	9a	10a	11c	12c
C1	200.2	216.3	210.3	209.9	204.0	203.7	200.1	203.4	72.8	72.8
C2	127.8		32.0	39.6	127.9	129.0	127.8	129.2	38.4	38.4
C3	142.8		26.9	77.5	147.7	139.7	142.8	139.9	74.8	74.3
C4	66.1	204.2	72.2	75.1	68.2	36.7	66.2	36.9	39.6	39.6
C5	78.1	60.4	65.8	65.0	80.3	73.7	78.1	73.5	139.8	139.7
C6	66.5	67.4	58.3	60.4	75.2	56.3	66.7	56.5	124.4	124.4
C7	39.3		29.2	31.2	38.2	57.0	39.4	57.5	32.7	32.8
C8	35.2		31.0	29.4	35.1	36.0	35.1	35.7	32.5	32.7
C9	45.3	41.8	42.5	42.8	46.2	35.2	45.8	35.2	41.8	42.0
C10	57.1	52.3	50.0	50.4	57.4	50.9	57.2	51.2	42.4	42.6
C11	22.4		21.1	21.6	24.0	21.6	22.7	23.4	21.0	20.9
C12	31.6		27.3	39.1	40.5	32.7	39.0	40.6	40.1	40.1
C13	48.3		42.3	42.7	44.1	48.7	43.2	44.1	43.2	43.3
C14	49.6		55.9	56.0	56.6	45.9	55.3	52.1	56.8	56.9
C15	23.3		23.8	24.3	25.2	22.9	24.0	22.0	27.7	27.7
C16	36.5	27..0	38.7	27.3	28.2	36.6	27.2	21.9	25.0	25.0
C17	84.8	51.6	51.4	51.9	53.0	84.6	51.8	54.6	52.5	52.5
C18	14.8	12.0	11.3	11.6	10.5	15.1	11.8	14.0	12.2	12.1
C19	9.9	13.2	15.0	15.7	12.2	14.7	9.9	15.0	20.1	19.9
C20	42.6	38.7	38.1	38.8	40.3	42.9	38.7	75.3	39.5	39.5
C21	9.4	13.4	13.3	13.4	13.6	9.5	13.3	21.3	14.0	13.9
C22	78.9	78.8	78.2	78.8	80.1	78.7	78.6	81.2	78.9	78.7
C23	32.9	29.1	28.9	29.8	30.7	32.4	29.8	31.9	30.4	29.9
C24	154.2	152.7	152.0	153.0	157.8	150.5	152.7	149.1	154.3	157.4
C25	125.2	126.0	125.2	125.7	120.4	121.4	125.7	122.2	127.8	124.5
C26	167.0	167.0	167.0	167.1	168.5	167.2	166.9	166.3	166.8	166.4
C27	57.3	57.5	57.5	57.5	56.3	12.4	57.4	12.7	56.7	63.9
C28	20.0	20.0	20.0	20.1	20.2	20.5	20.0	20.8	21.0	20.5
Glc-1’									103.7	103.3
2’									75.6	75.8
3’									78.8	79.1
4’									71.8	71.9
5’									77.4	78.8
6’									70.2	63.0
Glc-1”									106.0	105.4
2”									75.5	75.7
3”									78.9	79.1
4”									72.0	72.1
5”									78.7	79.0
6”									63.1	63.2
C3-O-CH3					56.86					

a In $CDCl_3$, δ (ppm) 125Hz;

b In CD_3OD, δ (ppm) 125Hz;

c In pyridine-d_5, δ (ppm) 125Hz;

3.5 Experimental

Optical rotations were obtained on a Rudolph Research Analytical Autopol IV Automatic polarimeter. Melting points were recorded using a MPA100 OptiMelt Automated Melting Point System (Stanford Research Systems, Sunnyvale, CA). UV data were acquired using an Agilent Technologies 1200 HPLC (Diode Array Detector). HPLC was conducted on Agilent Technologies 1200 series system. IR data were obtained using a Thermo Nicolet Avatar 360 FT-IR instrument. NMR spectra were recorded on either a Bruker DRX-400 with a qnp probe or on a Bruder AV-500 with a cryoprobe. ^{1}H and ^{13}C spectra were recorded using the residual protonated signal in the $CDCl_3$ solvent (δ_H 7.24) or the central peak of the $CDCl_3$ triplet (δ_C 77.00) as the internal standard. High resolution mass spectrometry data were collected on a LCT Premier time of flight mass spectrometer (Waters Corp., Milford, MA). Normal phase TLC was performed on Sorbent Technologies Silica G TLC plates (200 μm, w/UV 254) using the solvent system DCM-EtOAc-MeOH (1:8:1), and reverse phase TLC was performed on Sorbent Technologies C18 TLC plates (150 μm, w/UV 254) using H_2O-MeOH (1:1). Spots were visualized using UV light (254 nm) and spraying with vanillin-sulfuric acid reagent.

IV. LARGE-SCALE ISOLATION OF WITHAFERIN A FROM ROOTS OF *WITHANIA SOMNIFERA*

4.1 Introduction

Our interest in anti-proliferative activity of natural products and namely of withaferin A, led us to determine the chemistry and mechanisms responsible for its activity (see Chapter II). As follow–up studies will be necessary to further evaluate the potential of application of withaferin A for future translational studies in head and neck squamous carcinoma, we developed a large-scale isolation method for withaferin A for the future clinical studies.

We did not get sufficient quantities of withaferin A for the planned pre-clinical studies from an organic plant extract of *W. somnifera* purchased from PhytoMyco Research Corporation (discussed in chapter III). We considered, therefore, the purchase of roots of *W. somnifera* as our new source for the isolation of gram quantities of withaferin A. Roots and leaves of *W. somnifera* are traditionally used plant parts with different medicinal properties and uses in the Ayurvedic system of medicine. The powdered root of *W. somnifera* is also used as a nutrient and for health restoration to give energy and vigor. Reported HPLC analysis shows that roots have, in general, larger quantities of withanolides as compared to stems.[39]

4.2 Plant Material

The root powder (130 kg) of *W. somnifera* was purchased from Kalyx.com, Tamaqua, Pennsylvania in November 2009. The new plant material was accompanied by a certificate of analysis which has been deposited in the natural product chemistry lab at The University of Kansas for future reference.

4.3 Isolation of Withaferin A

Several batches of powdered roots of *W. somnifera* (30 Kg) were each extracted at room temperature for one week using 60 L of MeOH. After filtration, the plant filtrate was concentrated under reduced pressure using a rotary evaporator to get 600 g of a brown and sticky residue. This process was repeated one additional time using the same conditions to produce an additional 400 g of plant extract.

The total plant extract (1 kg) was divided into ten portions and each portion (100 g) was dissolved in 1.5 L of water to form a suspension. The water layer was placed in a large separatory funnel to which 1.5 L of hexane were added in order to remove the non-polar lipophilic organics such as fatty acids. The hexane layer was subjected to two additional liquid-liquid partitions. The hexane layers were combined and concentrated under reduced pressure to obtain a total of 110 g of a crude hexane extract. The water layers were each further placed in the separatory funnel to which 1.5 L of EtOAc were added in order to extract organic compounds of mid-polarity. The extraction process was repeated twice and the organic layers were combined and concentrated under reduced pressure to yield a total of 120 g of a crude EtOAc extract. Subsequently, the water layers were each extracted with BuOH three times (1 L × 3) to afford a

total of 220 g of a crude BuOH extract, which contained most of the polar organics. After all the described extraction procedures were completed, the aqueous layers were discarded.

Thin layer chromatography (TLC) of the hexane, EtOAc and BuOH extracts were co-chromatographed along with pure withaferin A as a standard reference compound. The TLC profiles showed that most of withaferin A was present in the EtOAc extract. As the isolation of pure withaferin A was the main focus of this project, we concentrated our efforts on its isolation from the EtOAc extract. TLCs were performed on Sorbent Technologies Silica Gel TLC plates (200 μm, w/UV 254) using the solvent system acetone-CH_2Cl_2 (1:4) and vanillin-sulfuric acid reagent as a visualization agent. Based on the TLC results, we decided to perform large scale (120 g of crude sample) column chromatography using silica gel (12~26 μm) and acetone-CH_2Cl_2 (1:9) as eluent.

The EtOAc extract (120 g) was dissolved with 1 L of EtOAc and added to 160 g of silica gel (12~26 μm). The mixture was dried under reduced pressure to afford yellow fine free-flowing powder. This EtOAc extract-silica gel mixture was subjected to passage over a silica gel column (column size: 150 mm × 600 mm; silica gel: 2 kg, 12~26 μm, Sorbent Technologies), which was then eluted sequentially with acetone- CH_2Cl_2 (1:9) to yield 31 fractions (Fractions 1-31; **Table 4.1**). TLC of each fraction was conducted during this process in order to monitor for the presence of withaferin A. Fractions 15-19 were shown to contain withaferin A and these were combined and dried under reduced pressure to afford 6.1 g of a yellow sticky residue.

The withaferin A-containing sample was placed in a pear shape flask and was dissolved in 20 ml of $CHCl_3$ and an equivalent amount of MeOH was added drop by drop. A piece of aluminum foil was placed over the flask and 5-8 small holes were punched to allow for a slow evaporation of the solvent at room temperature. After two weeks, light yellow and transparent crystals of withaferin A (4.5 g, 90% purity) were formed. In order to obtain a purer withaferin A, a second crystallization process was performed using the exact same method. After two weeks, colorless transparent crystals of withaferin A (4.0 g, 95% purity) were obtained.

Table 4.1. Fractions 1-31: Weights and Yields profile.

Fractions	Wt. (in g)	%Yield[a]
1	0.4	0.3
2	0.7	0.6
3	1.9	1.6
4	6.2	5.2
5	5.7	4.8
6	4.7	3.9
7	3.9	3.3
8	6.1	5.1
9	4.1	3.4
10	2.8	2.3
11	2.7	2.3
12	2.9	2.4
13	2.1	1.8
14	1.0	0.8
15	0.3	0.3
16	2.2	1.8
17	1.6	1.3
18	1.2	1.0
19	0.8	0.7
20	2.2	1.8
21	2.3	1.9
22	2.6	2.2
23	1.7	1.4
24	2.3	1.9
25	2.3	1.9
26	1.9	1.6
27	3.2	2.7
28	2.4	2.0
29	1.3	1.1
30	0.9	0.8
31	1.2	1.0

[a]As a percentage of the EtOAc extract (120.0 g)

4.4 Identification of Withaferin A

After recrystallization, the colorless transparent crystals (4.0 g) were identified as withaferin A (compound **1** in Chapter II) by ^{1}H NMR and co-chromatograph with compound **1** isolated from *Vassobia breviflora.*

4.5 Experimental

NMR spectra were recorded on a Bruker DRX-400 with a qnp probe. 1H and 13C spectra were recorded using the residual protonated signal in the CDCl3 solvent (δH 7.24) or the central peak of the CDCl3 triplet (δC 77.00) as the internal standard. Normal phase TLC was performed on Sorbent Technologies Silica G TLC plates (200 μm, w/UV 254) using the solvent systems CH_2Cl_2-EtOAc-MeOH (1:8:1) and acetone- CH_2Cl_2 (1:4). Spots were visualized using UV light (254 nm) and by spraying with vanillin-sulfuric acid reagent.

V. SUMMARY AND CONCLUSIONS

The research described here illustrated the isolation, characterization and biological activities of withanolides from the Argentinean plant *Vassobia breviflora* and the Indian plant *Withania somnifera.* The primary goal of this research was for anti-cancer drug lead discovery from medicinal plants. Following an introductory chapter describing the background and motivation for research into natural products, chapter 2 demonstrated how bioassay guided fractionation can lead to the identification of anti-proliferative agents. A total of 200 Latin American plants were evaluated for their cytotoxic activities. From one of the most active plants, *Vassobia breviflora*, the anti-proliferative active compound withaferin A (**1**) was isolated, along with another withanolide, viscosalactone B (**2**). The later biological study provided a possible anti-proliferative mechanism for withaferin A. In addition, three analogues, 2,3-Dihydrowithaferin A (**1_a**), 2,3-dihydro-27-deoxywithaferin A (**1_b**) and diacetylwithaferin A (**1_c**), were semi-synthesized from withaferin A for the anti-proliferative SAR studies.

Chapter 3 used a series of chromatographic separations and spectroscopic experiments to separate and identify 10 additional withanolides, 6α-chloro-5β,17α-dihydroxywithaferin A (**3**), (22*R*)-5β-formyl-6β,27-dihydroxy-1-oxo-4-norwith-24-enolide (**4**), 2,3-dihydrowithaferin A (**5**), 3-methoxy-2,3-dihydrowithaferin A (**6**), 2,3-didehydrosomnifericin (**7**), withanone (**8**) and 6α-chloro-5β-hydroxywithaferin A (**9**), withanolide A (**10**), withanoside IV (**11**) and withanoside X (**12**), from *Withania somnifera*. During this study, a new compound 6α-chloro-5β,17α-

dihydroxywithaferin A was isolated and (22*R*)-5β-formyl-6β,27-dihydroxy-1-oxo-4-norwith-24-enolide was found in *W. somnifera* for the first time. A method for large-scale isolation of withaferin A was presented in chapter 4. This method was developed to isolate four grams of pure withaferin A from 30 kilograms of *W. somnifera*.

This thesis described many natural products chemistry techniques including 1D and 2D NMR, mass spectroscopy, and a number of chromatography experiments. The compound **3** from *W. somnifera* was a novel withanolide with an unusual chlorine substitution on the B ring. The structure of this compound was first elucidated based on NMR, then confirmed by single crystal X-ray crystallography.

The biological studies demonstrated that withaferin A had anti-proliferative and apoptotic inducing properties on HNSCC cells. The growth inhibitory effect of withaferin A in HNSCC cells was accompanied by a G2/M arrest in cell cycle. All withanolides in this study were evaluated in assays for anti-proliferative activities against HNSCC cells. Results obtained in these studies suggested the importance of some structure features of withaferin A for it observed anti-proliferative activity. The α,β-unsaturated A ring and 5,6-epoxy-4-ol are important for its anti-proliferative activity.

Recent studies beyond the scope of this thesis were performed by our collaborators, Dr. Mark Cohen, at KU medical center. *In vitro* tests indicated that withaferin A was an Hsp90 modulator,

with inhibition of HSF-1, Akt and caspase-3 expression and cleavage of PARP.[40] *In vivo* animal studies by also demonstrated that withaferin A was a RET (rearranged during transfection) inhibitor and had significant efficacy against medullary thyroid cancer.[41] Recent published withaferin A anticancer mechanism studies reported that withaferin A induces endoplasmic reticulum (ER) stress[42] and inhibition of the NF-κB signaling pathway.[43] Based on the reported biological studies, withaferin A and its analogues are the potential drug leads for the treatment of cancer[30], neurodegenerative[33], inflammatory[35], and cardiovascular[32] diseases.

The work presented here demonstrated a natural product approach to discovering potential botanical new drug leads, which starts with a library of plant extracts to one plant, then to one active compound, and to a series of analogues at the end. The work also showed how to optimize the small-scale isolation method and apply it to a large-scale isolation for sufficient materials for pre-clinical studies. This work was published in two papers[22][38] and also contributed to a patent application.[44] This thesis emphasized the importance of natural products to the discovery of biological active agents, and concluded that natural products have long been and will continue to be important to the anti-cancer drug research. The future direction of this research will concern on the total synthesis of withanolide-type compounds, the searching of novel withanolides from other plant species, the elaborate anti-proliferative SAR studies and the further anti-proliferative clinical studies of withaferin A and its analogues.

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